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HEPATIC ENCEPHALOPATHY

The role of blood-brain barrier derangements, ammonia,
amino acids and neurotransmitters



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HEPATIC ENCEPHALOPATHY. The role of blood-brain barrier derangements, ammonia, amino acids and neurotransmitters

Abstract

Increased blood-brain transport of large neutral amino acids (LNAA) in liver failure may contribute to hepatic encephalopathy (HE). The mechanism of this increased blood-brain transport is unclear.

In dogs with portacaval anastomosis (PCA) a good correlation was found between the glutamine concentrations and the concentrations of the other LNAA in the central nervous system. In rats with PCA and in normal rats infused with ammonia the LNAA in brain increased. Inhibition of the glutamine synthesis with methionine sulfoximine almost normalized the LNAA brain concentrations. Arterial injections of radioactive labelled LNAA (leucine and phenylalanine) were used to measure the rate of blood-brain transport in normal rats infused with ammonia. Ammonia infusion increased the brain uptake of leucine and phenylalanine. Pretreatment with methionine sulfoximine prevented these changes completely. These results demonstrate that an intact glutamine synthesis is important for blood-brain transport of LNAA in conditions with hyperammonemia. It is suggested that the LNAA transport across the blood-brain barrier is mediated by a mobile carrier mechanism whose rate of transport is dependent on the substrate loading of the carrier.

Seven patients with liver cirrhosis and a history of HE were given ammonia orally. Despite high ammonia levels in the cerebrospinal fluid (CSF), there was an increase in the glutamine and the other LNAA in the CSF in one patient only. This patient had the most pronounced HE of the patients studied. There was a good correlation between CSF glutamine and other LNAA suggesting that also in humans glutamine is involved in LNAA transport into brain.

Branched chain amino acids have been suggested as a treatment of encephalopathy in liver failure. Infusion of BCAA into hepatectomized rats normalized the brain concentrations of the other LNAA, and reduced the indoleamine concentrations in brain below normal levels, but did not improve survival. In rats with PCA, infusion of ammonium salts with addition of BCAA prolonged life and reduced the concentrations of ammonia in plasma and in brain, compared to rats with PCA infused with ammonium salts alone. BCAA may protect against hyperammonemia in chronic liver failure by stimulating the peripheral detoxification of ammonia.

Key words
Hepatic encephalopathy, ammonia, glutamine, blood-brain barrier, amino acids, neurotransmitters

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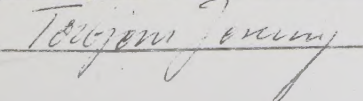
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HEPATIC ENCEPHALOPATHY

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amino acids and neurotransmitters

by

Torbjörn Jonung

Lund 1985

This thesis is based on the following papers:

- I Factors influencing the concentrations of the large neutral amino acids in the brain and in the CSF of dogs after portacaval anastomosis. Ramzy A, Jonung T, Herlin P, Chance W T, James J H, Fischer J E. Submitted for publication J Surg Res.
- II. Methionine sulfoximine prevents the accumulation of large neutral amino acids in brain of portacaval-shunted rats. Rigotti P, Jonung T, Peters J C, Howard J H, Fischer J E. J Neurochem 44 : 929-933, 1985.
- III. Methionine sulfoximine prevents the accumulation of large neutral amino acids in brain of hyperammonemic rats. Jonung T, Rigotti P, Jeppsson B, James J H, Peters J C, and Fischer J E. J Surg Res 36: 349 - 353, 1984.
- IV. Effect of hyperammonemia and methionine sulfoximine on the kinetic parameters of blood-brain transport of leucine and phenylalanine. Jonung T, Rigotti P, James J H, Brackett K, Fischer J E. Accepted for publication in J Neurochem 1985.
- V. Indole amines and amino acids in various brain regions after infusion of branched chain amino acids into hepatectomized rats. Jonung T, Ramzy A, Herlin P, James J H, Edwards L, Fischer J E. Eur Surg Res 17 : 83-90, 1985.
- VI. Effects of infusing branched chain amino acids and ammonium salts in rats after portacaval anastomosis. Rigotti P, Jonung T, James J H, Edwards L L, Peters J C, Fischer J E. Accepted for publication in Arch Surg, 1985.
- VII The effects on the plasma concentrations of amino acids, insulin and glucagon and on the CSF concentrations of amino acids and indoleamines after administration of ammonia to patients with liver cirrhosis. Jonung T, Jeppsson B, Herlin P, Nobin A, Hultberg B, Åslund U. In manuscript.

In the text the papers are referred to by their Roman numerals.

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INTRODUCTION

Liver disease is a leading cause of death in the western world. In the USA, only heart disease, stroke and cancer exceed liver disease as the death cause of both sexes (1). Hepatic encephalopathy (HE) is a major complication of severe liver disease. It involves changes in the personality, abnormalities in motor function, such as tremor, rigidity, hyperactive reflexes and, in advanced stages, decerebrated postural responses and altered levels of consciousness which may finally end in hepatic coma and death. The onset of hepatic coma may come abruptly as in fulminant liver failure following a viral hepatitis, or more slowly as in patients with chronic liver failure with spontaneous or surgical portal-systemic shunting in whom the personality changes and the other symptoms are more insidious and fluctuating. The etiology of HE is still unknown. Since liver disease is accompanied by a disturbed nitrogen metabolism, substances involved in the nitrogen metabolism have predominantly been suggested to be etiologic factors, such as ammonia and amino acids.

SOME ASPECTS ON CURRENT CONCEPTS OF THE ETIOLOGY OF HEPATIC ENCEPHALOPATHY

Ammonia

High concentrations of ammonia are found in patients (2,3,4,5) and in experimental animals with HE (6,7,8). Precipitation of HE by an acute ammonia load has been observed in patients with liver disease (4,9). Administration of ammonia in experimental animals produces symptoms similar to those found in humans with HE (10,11). It has been difficult to establish a quantitative correlation between the intensity of HE and the plasma ammonia concentrations. High ammonia concentrations in plasma are usually found in patients with HE but patients may remain in coma even after normalization of plasma ammonia levels (4). In liver failure, ammonia absorbed and produced by the gut escapes liver detoxification not only due to an impaired liver function but also to portal-systemic shunting (12). In brain and in peripheral tissues, ammonia is incorporated with glutamic acid to form glutamine (13,14). Under normal circumstances in humans, 50% of the ammonia is metabolized in the muscles (5). Muscle wasting is a well-known feature in patients with chronic liver failure, and the uptake of ammonia by the muscles is reduced in patients with

liver cirrhosis and muscle wasting, compared to those cirrhotics without a reduction in the muscle mass (15). Reduced uptake of ammonia into muscles and liver in patients with chronic liver failure probably results in increased exposure of the central nervous system to ammonia. It has been demonstrated that an ammonia load in rats with PCA increased the brain concentrations of ammonia 50-100% compared to normal rats (6). In patients with liver cirrhosis, the rate of ammonia clearance from the blood by the brain was directly proportional to the arterial concentrations of ammonia (5). In brain, ammonia is rapidly detoxified to glutamine, probably by the glia cells surrounding the cerebral capillaries (13). This process is exceedingly rapid. Glutamine can be further metabolized by transamination with an alfa-keto acid to yield alfa-ketoglutarate and the corresponding amino acid (16). High concentrations of glutamine and glutamate have been found in patients with HE, and no other parameters show a better correlation with the grade of HE (16,17).

A close link between the ammonia detoxification process and the acute toxic effect of ammonia seems to exist. Inhibition of the glutamine synthesis by methionine sulfoximine, an inhibitor of glutamine synthetase, has been found to prolong survival after an acute ammonia load in normal rats (18). The mechanism by which ammonia affects the brain metabolism is obscure. Ammonia detoxification in brain consists of a two-step process: glutamic acid is formed from alfa-ketoglutarate by a reductive amination and subsequently amidated to glutamine. This process requires ATP and could deplete the tricarboxylic acid cycle in brain of its alfa- ketoglutarate (19). Hindfelt et al (10,20) studied the effect of an acute ammonia intoxication on normal rats and on rats after PCA but could not find reduced alfa-ketoglutarate or ATP concentrations before onset of coma. They concluded that cerebral dysfunction in chronic, relapsing ammonia intoxication or in acute ammonia intoxication is not a result of primary energy failure.

In experimental animals with hepatic failure of different etiologies and in ammonia-infused normal animals, swelling of the astrocytes surrounding the brain capillaries has been reported (21). The same changes have been observed also in patients dying of HE (21). This swelling is probably an effect of hyperammonemia, and it may alter the metabolism of the astrocytes and thereby also affect the metabolism of the neurons and contribute to HE (11).

There have been two arguments against ammonia as the major pathogenetic agent in HE: 1/ Acute ammonia intoxication in normal experimental animals results in a hyperkinetic preconvulsive or convulsive state (10,22). The same clinical picture is found in humans with inherited metabolic disorders resulting in acute hyperammonemia. This is in contrast to the hypokinetic picture seen in rats with PCA after an acute ammonia load (10) and in

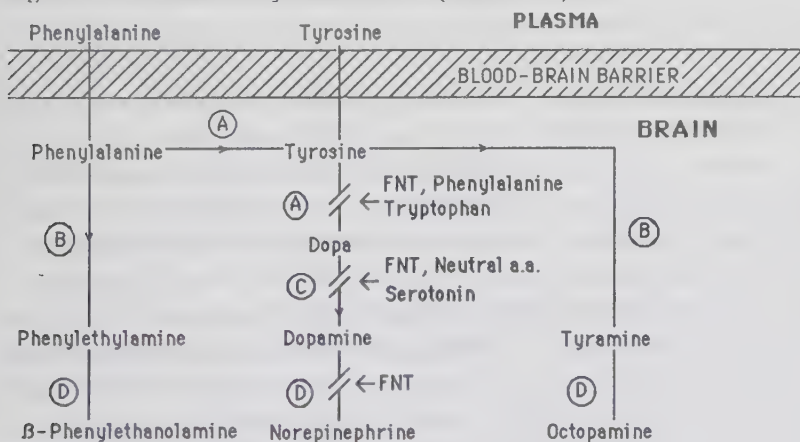
humans with hepatic coma. 2/ It has been difficult to show a direct correlation between the intensity of HE and the plasma ammonia concentrations (4). Patients in hepatic coma can have normal concentrations of ammonia in plasma. Taken together, the above mentioned findings suggest that other factors, such as derangements in the blood-brain transport and altered amino acid and neurotransmitter metabolism, may play an important role in the development of HE.

Amino acid and neurotransmitter hypothesis

Alterations in plasma and brain amino acid concentrations in liver failure in both humans and experimental animals have been described by many investigators. In humans with chronic liver cirrhosis, the typical changes in the concentrations of the large neutral amino acids (LNAA) in plasma are a decrease in the branched chain amino acids (BCAA) and an increase in tyrosine, phenylalanine, methionine and free tryptophan (23,24). The mechanisms underlying these changes are not obvious. It has been suggested that the amino acid changes may be due to the catabolism following liver insufficiency with increased glucose formation from amino acids, secondary to hyperglucagonemia and hyperinsulinemia. Increased plasma concentrations of tyrosine, phenylalanine, tryptophan and methionine could be a result of impaired liver metabolism, and low concentrations of BCAA a result of increased metabolism of these amino acids by muscles or by fat (25,26). However, the importance of high plasma concentrations of glucagon and insulin for the plasma amino acid changes is uncertain. High plasma glucagon concentrations in patients with chronic liver failure correlate with the degree of portal-systemic shunting, but not with liver function (27,28). In patients with spontaneous portal-systemic shunting and normal liver function, no increase in the insulin concentrations were found although the plasma concentrations of BCAA were reduced (29). In patients with liver cirrhosis, the effect of insulin on the peripheral BCAA uptake was reduced (30). These findings demonstrate that other factors than insulin contribute to the low plasma levels of BCAA in liver failure. One factor could be high plasma concentrations of ammonia. Ammonia detoxification consumes α -ketoglutarate which is supplied from the BCAA (14). In accordance with this, Marchesini et al demonstrated a decrease in the plasma concentrations of BCAA in humans with liver cirrhosis after ammonia tolerance tests (9). These results are not in agreement with the results in our study (VII).

In the central nervous system (CNS), an accumulation of LNAA is found in experimental animals after PCA (31), after hepatectomy (32,33), and in humans with HE (24). This may be of importance for the development of HE since some of the LNAA are involved in the neurotransmitter synthesis. Tyrosine and phenylalanine are precursors of the catecholamines, and tryptophan is a precursor of serotonin. The neuronal pathways of these neurotransmitters are supposed to influence the muscle tonus and the sleep/wake cycle. Fischer and Baldessarini (34) suggested that some of the neuropsychiatric symptoms in HE could be explained by an accumulation of false adrenergic neurochemical transmitters, primarily octopamine and phenylethanolamine, in the nerve terminals instead of the original neurotransmitters norepinephrine and dopamine. High brain concentrations of phenylalanine may inhibit hydroxylation of tyrosine to dopa. Excess of phenylalanine and tyrosine is decarboxylated to phenylethylamine and tyramine, respectively, which are oxidated by dopamine beta-oxidase to the potentially false neurotransmitters beta-phenylethanolamine and octopamine. High brain concentrations of tryptophan, beta-phenylethanolamine, octopamine and serotonin could further inhibit the metabolism of tyrosine to norepinephrine by competitive inhibition of the different enzymes (35)(Fig 1).

Figure 1. Catecholamine Synthesis in Brain (reference 35)



A = tyrosine hydroxylase; B = aromatic amino acid decarboxylase; C = dopa decarboxylase; D = dopamine β-oxidase; FNT = β-phenylethanolamine.

In support of this hypothesis, high concentrations of octopamine have been found in rats with portacaval anastomosis (36). In dogs with PCA, increased concentrations of octopamine, phenylethanolamine (37), and tyramine (38) have been observed in plasma and in CNS. Also in humans with liver cirrhosis, high plasma concentrations of octopamine and phenylethanolamine have been reported (39). Reduced brain concentrations of norepinephrine have been found in rats with liver ischemia (40), in rats after hepatectomy (32), and in dogs after PCA (38). In humans, however, increased concentrations of norepinephrine have been observed in the cerebrospinal fluid (CSF) (41). High concentrations of serotonin, its precursor tryptophan and its metabolite 5-hydroxyindoleacetic acid (5-HIAA) have been reported in post mortem studies of brains from patients dying of HE (42), in rats after PCA (43), and in rats in acute hepatic failure following two-stage hepatic devascularization (44).

Local variations of the catecholamine and the serotonin neuronal systems have been demonstrated (45). In accordance with this, regional variations of amino acid and neurotransmitter concentrations in brain have been reported in patients dying of HE (42), and in experimental animals with liver failure (43,44). Local changes of the amino acids and neurotransmitters may be of importance for development of HE. There is, however, little information available whether changes of the LNAA and the neurotransmitters in the different brain regions parallel with the grade of HE and the results of different treatments.

Another theory involving neurotransmitter changes was introduced by Schafer and Jones in 1982 (46). They proposed that gamma-aminobutyric acid (GABA), the principal inhibitor of the neurotransmitters in brain, could be of importance for the pathogenesis of HE. Increased numbers of GABA receptors have been found in rats with hepatic coma provoked by galactosamine. High levels of GABA-like activity have been demonstrated in humans with HE (47), but the correlation between HE and the GABA-like activity was poor. Increased blood-brain transfer of alfa-aminoisobutyric acid (AIB) was observed in rabbits after galactosamine injection (46). However, increased blood-brain permeability in fulminant hepatic failure has been reported for other substances and does not "single out" GABA. Visual evoked potentials (VEP) in rabbits with fulminant hepatic failure, due to galactosamine, showed the same pattern as in comas induced by pentobarbital and diazepam, which activate the gamma-aminobutyric acid-ergic neural mechanisms (46). The fact that two neurological conditions have the same VEP does not prove they share the same etiology. The role of GABA receptors is mainly investigated in acute liver failure in rabbits after galactosamine injection. It is, however, not clear to what extent the observed results in

the experiments are an effect of galactosamine itself. For the validity of the GABA hypothesis, the role of GABA must be investigated and confirmed in other models of HE than after galactosamine injection, such as after PCA and hepatectomy, and in other experimental animals than in rabbits.

Although changes of the neurotransmitter concentrations and of the GABA-receptors have been shown in liver failure, we do not know what importance these changes have for the development of HE.

Derangements of blood-brain transport of large neutral amino acids in liver failure

The transport of nutrients, and thus their availability to the brain, is strictly regulated by the blood-brain barrier. Its anatomical structure is probably made up of the capillary endothelial cells of the brain (48). These cells are connected with tight junctions and, as a consequence, all transport of nutrients must go through the endothelial cells. Increased permeability of the blood-brain barrier could permit entrance of toxic substances into brain and increase the availability of certain nutrients that may be harmful in excessive amounts.

Blood-brain transport of LNAA has attracted special interest because some of them are precursors of neurotransmitters, and because several pathological states, both genetic and acquired, result in altered levels of these amino acids. Studies of blood-brain transport of LNAA have been performed predominantly in rats.

Brain uptake of LNAA seems to occur by a system of two components (49). One of these is saturable (49,50), stereospecific (51), and exhibits competition among the LNAA for uptake into brain (52). This component follows the Michaelis-Menten kinetics and resembles in its properties the L-system as described by Christensen (53). The other component seems to be non-saturable even at very high concentrations (49,50). In normal rats after a meal, the brain concentrations of the LNAA are proportional to their calculated rates of influx from the plasma (54), but rats with PCA have higher brain concentrations of LNAA than could be expected from the changes in the plasma LNAA concentrations (31,55,56). In conditions with fulminant hepatic failure after hepatectomy (32,33) and after PCA in rats (31,56), increased brain uptake of LNAA has been demonstrated. Increased brain uptake of some of the LNAA has also been observed in humans with liver cirrhosis (57).

Fulminant hepatic failure after hepatectomy in rats (33) results in increased permeability of the blood-brain barrier not only for LNAA but also for inulin and sucrose, substances to

which the blood-brain barrier is normally virtually impermeable. Increased permeability to inulin has been shown in rats 6 weeks after PCA (58), but not after two weeks (31). Electron microscopic examination has demonstrated that these changes in the permeability of the blood-brain barrier is probably not due to a disruption of the tight junctions between the endothelial cells (59). Swelling of the astrocytes surrounding the brain capillaries in liver failure may play a role in the function of the blood brain barrier. Such changes of the astrocytes are reported in humans, in experimental animals in liver failure, and in animals after ammonia infusion (11,21).

James et al (60) observed an excellent correlation between high brain levels of glutamine and LNAA in rats after PCA. From these data they suggested that increased blood brain transport of LNAA is a result of high brain concentrations of glutamine, the detoxification product of ammonia. This hypothesis linked hyperammonemia to altered amino acid pattern in brain and thereby also to altered neurotransmitter function.

Glutamine and the other LNAA compete for the same blood-brain transport system for entry into brain (52,61). It is a concentration dependent transport system with capability of exchange transport (53). High brain concentrations of glutamine may inhibit efflux of LNAA out of brain (60,62) resulting in an accumulation of LNAA in brain. The concentration gradients of the amino acids across the blood brain barrier may further accelerate exchange transport of the LNAA into brain. Infusion of ammonium salts (63) and intraperitoneal injection of urease (64) into normal rats result in high brain concentrations of glutamine and LNAA with only a slight effect on plasma amino acid concentrations. Increased brain uptake of tryptophan is also demonstrated after infusion of ammonium salts (65) and injection of urease (64). Whether this is due to a direct effect of ammonia or of glutamine is disputed. Mans et al (65) found no correlation between high brain glutamine concentrations and the brain uptake index for tryptophan after infusion of ammonium salts. They demonstrated that an increase in the brain glutamine concentrations came before an increase in the brain uptake index of tryptophan. One explanation of their observation could be a delay of the effect of glutamine and ammonia on the blood-brain transport system. Other critics argue that glutamine is only a weak competitor for the LNAA transport system and that it would be unlikely for glutamine to stimulate the LNAA blood-brain transport.(66). This is contradicted by the fact that studies in isolated bovine capillaries have demonstrated that preloading of the microvessels either with glutamine or with another LNAA, methionine, stimulated the uptake of LNAA into the microvessels (67). Consequently, it is not clear whether the increase in the brain uptake of the LNAA measured in vivo is a cause or an effect of high brain concentrations of LNAA. It is also uncertain what effect ammonia

itself can have on the concentrations of these amino acids in brain. To clarify the role of ammonia and of glutamine in the LNAA transport across the blood-brain barrier one needs to inhibit the glutamine synthesis in experimental animals in conditions with hyperammonemia and study the effect on the blood-brain transport of the LNAA. Moreover, the effects of ammonia on the blood-brain transport of LNAA and on the concentrations of LNAA in the central nervous system in humans are unknown.

Branched chain amino acids and treatment of hepatic encephalopathy

The amino acid neurotransmitter hypothesis presented by Fischer and Baldessarini suggests that correction of the disturbed plasma amino acid pattern and thereby also correction of the disturbed brain amino acid and neurotransmitter pattern would be beneficial in the treatment of HE. Studies on experimental animals with PCA and humans with liver failure resulted in the proposal that treatment with BCAA could: 1/ correct the abnormal plasma amino acid profile and stimulate the protein synthesis, 2/ reduce the brain concentrations of phenylalanine, tyrosine and tryptophan by competition for the same transport system and thereby also reduce the brain concentrations of "false" neurotransmitters, improve HE, and protect against it (8,23,37,68). Treatment with BCAA may also improve the nutritional status in patients with chronic liver failure by permitting a higher protein intake without worsening the HE and, in doing so, reduce the catabolic state of these patients (68). During the last few years, several prospective randomized trials of the effect of parenteral and enteral BCAA treatment in patients with liver failure have been published. The results have been far from unifying as to the question of the beneficial effect of BCAA on the treatment of HE (Table 1).

The different conclusions of the trials have focused the interest on the different designs of the studies and how future studies should be performed to give more conclusive results. One fault is that the overall number of patients treated in these studies is small. From a statistical point of view it is only in the US multicenter-trial that the differences between the groups are big enough to draw a definite conclusion (75). The etiology of the liver diseases, the precipitating factors, the exclusion criteria, and the grade of HE of the patients when entering the trials must be considered when the results are evaluated. For example, the outcome of an alcoholic cirrhosis is affected by the recent drinking habits. The percentage of alcoholics included in the studies have varied from about 30% (2) to 100% (73). In no study, the outcome has been related to the drinking habits. For future trials, the

Table 1. BCAA in Hepatic Encephalopathy

TRIAL	PATIENTS (n)	TREATMENT	CONTROL	OTHER TREAT- MENT	OUTCOME
Rossi-Fanelli (2)	34	BCAA + gluc	lact + gluc	--	similar results
Fiaccadori (69)	48	BCAA + gluc	lact + gluc	* BCAA + lact	BCAA pos
Cerra (70)	22	BCAA + gluc	neom + gluc	--	BCAA pos
US-mult (71)	59	BCAA + gluc	neom + gluc	--	BCAA pos
Strauss (72)	29	BCAA + ?	neom + ?	--	BCAA shorten coma
Glud (73)	20	BCAA + gluc	gluc	conventional therapy	BCAA may shorten HE
Michel (74)	47	BCAA + gluc +lipid	standard AA +gluc + lipid	--	similar results
Wahren (3)	50	BCAA + gluc + lipid	gluc + lipid	**neomycin+lact	similar results

Numbers within parenthesis refers to reference numbers. gluc = glucose; lact = lactulose; pos = positive results; neom = neomycin; AA = amino acid solution; mult = multicenter-trial. * a third treatment group; ** in only eight patients.

outcome of the treatment should be related to the etiology of the liver disease. Bleeding and infections were the major precipitating factors in two trials (2,3). In another trial 45% of the HE was a reaction to an operative trauma (70). If the treatment of the precipitating factors is unsuccessful, the patients are likely to die whatever is done. The outcome of a trial should therefore also be related to the success of the treatment of the precipitating factors. The patients have had HE grade II - IV when entering the trials. Patients with HE grade II have a high spontaneous recovery. If these patients are not excluded, the number of patients who recover will be large, a large number of patients will be needed in the studies, and it will be difficult to evaluate differences between the treatment groups. On the other hand, many of grade IV patients are so sick that they will die whatever is done. If these patients are included in the trials, the mortality rate will be too high and there will be difficulties in evaluating the results. To solve this problem, some investigators did not enter the patients into the studies until they had been in coma for about 24 hours (3) or had not responded to

"traditional care" within 48 hours (70). There were also great differences in the length of the treatment, which varied from 2-4 days (2) to 4-14 days (70). If BCAA have a positive effect on the protein metabolism, this effect will probably not be demonstrated if the treatment period is too short. One fundamental difference between the two largest trials, the BCAA positive American trial (71) and the BCAA negative Swedish-French one (3), was the type of amino acid solutions. In the Swedish-French trial a mixture of the three BCAA (70% leucine) was given, in the American trial BCAA were administered together with a mixture of other essential and non-essential amino acids. Whether or not the differences between the trials can be explained by the differences in amino acid solutions remain to be demonstrated. To be able to compare future investigations, the same amino acid solution should be used. If the BCAA treatment is planned to continue for some time, a balanced amino acid solution is probably to be preferred. Due to inhibition of transport into brain, a solution containing only BCAA would prevent other LNAA from entry into brain and deplete the brain of other essential amino acids.

In vitro studies have shown that the BCAA stimulate synthesis and reduce degradation of muscle proteins (76,14). The effect of BCAA on nitrogen balance has been studied in muscle-wasted patients with chronic HE. Horst et al (77) compared a standardized enteral protein load to a standardized enteral protein load with addition of BCAA in 37 patients with at least HE grade II. They found that the addition of BCAA induced a positive nitrogen balance without worsening the HE. In another trial, Egberts et al compared casein and BCAA supplementations to the diet in 22 patients with minor HE (78). They found a positive effect of BCAA, compared to that of casein, on the HE and on the nitrogen balance. Thus, addition of BCAA to the diet seems to be well tolerated and could increase the total intake of protein in patients with liver failure without deterioration of their mental status.

BCAA may also reduce the plasma ammonia concentrations (79,80). In accordance with this, Marchesini et al have demonstrated that an ammonia load in patients with liver cirrhosis resulted in reduced plasma concentrations of BCAA (9). Rössle et al (81) found a decrease in the plasma and CSF ammonia concentrations after BCAA treatment with improvement of the HE. However, no control group was used in that study. The reduction in the ammonia concentrations was much less in plasma than in CSF. In a controlled trial Fiaccadori et al (69) found a significant reduction in the plasma ammonia concentrations compared to the control group, while Wahren et al (3) found a decrease in plasma ammonia concentrations with improvement of HE both in the control group and in the BCAA treated group. Other controlled trials could not find a reduction in plasma ammonia in either group

parallelly with improvement of HE (2,70). Measurements of ammonia in patients with HE have always resulted in great differences between studies. One explanation, except for differences in the design of the trials, is poor handling of blood samples for ammonia determination. Secondly, in order to avoid false negative ammonia results, arterial samples are to be preferred (4). In all the trials venous blood samples seem to have been used.

Further clinical and experimental studies are needed to clarify the mechanism and the interaction of ammonia and BCAA, for example by studying combined infusion of BCAA and ammonia.

Derangements of blood-brain transport of LNAA and of other substances are a frequent finding in liver failure. Increased permeability of the blood-brain barrier may expose the brain to toxic substances that could influence the brain metabolism and contribute to HE. High brain concentrations of glutamine, secondary to hyperammonemia, have been suggested to increase the blood-brain transport of LNAA and thereby increase the brain concentrations of these amino acids. In order to study the relationship between the LNAA concentrations in plasma and in the central nervous system we determined the LNAA concentrations in plasma, CSF and brain in dogs with PCA. To clarify the role of ammonia and glutamine in the brain concentrations and the brain uptake of LNAA, we wanted to investigate the effect of inhibition of the glutamine synthesis 1/ on the plasma and brain concentrations of LNAA in rats after PCA and in normal rats after infusion of ammonium salts 2/ on the kinetics of blood brain transport of leucine and phenylalanine in normal rats. We also wanted to study the effect of ammonia tolerance tests on the amino acids in plasma and CSF in patients with liver cirrhosis.

Altered plasma and brain amino acid pattern and, secondary to this, changes in the neurotransmission have been proposed to contribute to HE. Treatment with BCAA has been suggested to correct the amino acid imbalance in plasma and brain and thereby also correct the neurotransmission and improve HE. A connection between the ammonia and the BCAA metabolism has also been suggested in that BCAA could accelerate the detoxification of ammonia and protect against an acute ammonia load. Clinical studies of acute HE have given divergent results as to the effects of BCAA treatment. One purpose of this work was to observe the effects of BCAA treatment on amino acids, indole amines, and survival in acute liver failure after hepatectomy in rats. Another purpose was to study the effects of BCAA treatment on amino acids, survival, neurotransmitters and ammonia metabolism in rats with PCA after infusion of ammonium salts alone, and after infusion of ammonium salts and BCAA simultaneously.

METHODOLOGICAL CONSIDERATIONS

Animals

Rats: Male Sprague-Dawley rats from Charles River Laboratories, Wilmington, MA, were used in paper II-VI. The rats in paper II, IV, VI had an initial weight of 300-350 g, in paper III 270-340 g, in paper V 225-275 g. The animals were housed two per cage and the light was on between 7 a.m. and 7 p.m. Water and rat chow ad libitum (Ralston Purina Co., St. Louis, MO) were available at all times before the experiments.

Dogs: Mongrel dogs weighing 9.5-23.9 kg were used in paper I. The dogs were housed in individual cages. The animals were fed with chow (Ralston Purina Co., St Louis, MO) and received tap water ad libitum. The dogs were conditioned for two weeks before operation and were free from parasites and from kidney and liver diseases.

Experimental models

The rat with a portacaval anastomosis (PCA) is a commonly used experimental model to study metabolic changes after deviation of the portal blood flow around the liver. These rats develop hyperammonemia (7), astrocytosis (59) and alterations of the amino acids (31,56) similar to what is found in humans with chronic liver failure. Behavioural changes after portacaval anastomosis in rats have also been observed (82), but the rats do not go into coma. In order to bring the rats into metabolic coma one has to administer a toxic agent, such as ammonia. This is in contrast to dogs where the majority, after deviation of the portal blood flow, develop severe HE with coma and death. It may be questionable to draw clinical conclusions from studies in shunted animals, with a non-cirrhotic liver. In the absence of an acceptable model of liver cirrhosis in experimental animals portacaval shunt is, however, still a model for investigation of metabolic changes in HE.

In this study, portacaval shunt in dogs was used to follow amino acid changes as the HE progressed. Ammonia infused rats with portacaval anastomosis provided a model somewhat similar to what is found in patients with chronic portal systemic encephalopathy after an acute nitrogen load.

Total hepatectomy and liver devascularization are two models of acute hepatic failure in experimental animals . After liver devascularization, liver necrosis is a major source of the plasma amino acid changes (83). To study the effect of total liver failure on the peripheral tissues without the influence of a necrotic liver, total hepatectomy seemed to be a good model and was used in the present work.

Portacaval anastomosis

Rats: The portacaval shunts in study II,V and VI were performed under ether anaesthesia utilizing clean, but not sterile, technique. The abdominal wall was opened through a midline incision and the portal vein was dissected free. The pancreaticoduodenal vein was ligated and the end-to-side portacaval anastomosis was constructed with continuous 7-0 silk sutures. Before the abdominal wall was closed, 10-15 ml sterile saline was instilled in the peritoneal cavity. The perioperative mortality was about 5%. Food and water were available immediately after the operation. The portacaval shunts in the hepatectomy study (V) were sutured with 9-0 Ethilon® without ligation of the pancreaticoduodenal vein.

Dogs: The dogs in study I were anaesthetized with sodium pentobarbital . The abdomen was opened through a midline incision and the portal vein was dissected free. The end-to-side portacaval anastomosis was sutured with continuous 5-0®Prolene . The shunt had to be completed within 20 minutes as the dogs did not survive a longer clamping time of the portal vein. After the operation a 5% glucose in saline solution was infused for 24 h. Food and water were available from the second day after the operation. Penicillin and streptomycin were given as a single dose just before surgery. All dogs lost weight after the operation and the per cent weight loss at sacrifice was proportional to the grade of encephalopathy.

Dog experiment (study I)

In dogs with PCA blood samples were obtained preoperatively and weekly after the operation. Under sodium pentobarbital anaesthesia cerebrospinal fluid was obtained by cisternal puncture preoperatively and at 3 weeks intervals after the operation. Starting from the second postoperative day two observers independently rated the dogs for encephalopathy. The animals were clinically graded as follows.

Grade 0 Indistinguishable from normal (no animals were sacrificed in this condition).

Grade I Less lively and with delayed responses.

Grade II Hypersalivation; hyperactive movements; walking close to the walls with an unsteady gait.

Grade III Excessive salivation; sleeping most of the time; when forced to walk, walking "into" the walls; standing with great difficulty; very slow response to foot-pinch; coma.

At time of sacrifice the dogs were anaesthetized with pentobarbital, and blood and CSF samples were taken. The vault of the skull was removed by an electric saw and the brain disconnected at the junction with the spinal cord and rapidly transferred to ice-cold normal saline. The brain was then put on its dorsal surface on an ice-cold, saline soaked paper towel. Nine brain regions were isolated separately and in the following order: hypothalamus, diencephalon, cerebellum, medulla, pons, mesencephalon, cortex, caudate nucleus, and hippocampus. The brain samples were immediately frozen in liquid nitrogen and stored at -72° C until analysis.

Methionine sulfoximine

Methionine sulfoximine (MSO), an irreversible inhibitor of glutamine synthetase, was dissolved (50 mg/ml) in 0.15 M NaCl and injected intraperitoneally at a dose of 150 mg/kg. This dose of MSO caused no convulsion during the experiments (II,III and IV).

Methionine sulfoximine treatment in rats with portacaval anastomosis (study II)

12-14 days after PCA the rats received an intraperitoneal injection either of MSO or of diluent alone. 24 h later the rats were sacrificed by decapitation.

Infusion of ammonium salts and methionine sulfoximine treatment in normal rats (study III and IV)

The rats were infused for 24 h with ammonium salts (the same solution as in study VI) with or without treatment of methionine sulfoximine before start of the infusion. The infusion was delivered at a rate of 0.7 ml/h/100 g body weight. The infusion of ammonium salts made the rats drowsy after a few hours but did not cause convulsions.

Hepatectomy (study V)

The hepatectomy was performed using clean but not sterile technique in a three stage procedure under ether anaesthesia as described by Holmin et al (84):

(a) division of inferior vena cava above the renal veins

(b) 4 weeks later, end-to-side portacaval anastomosis without ligation of the pancreaticoduodenal vein

(c) 1 week later, removal of all liver parenchyma after ligation of the hepatic artery, common bile duct, and the inferior vena cava close to the diaphragm.

Control groups underwent either steps (a) and (b) and a sham operation at (c)(group C) or step (a) and sham operations at steps (b) and (c)(group D). After the third operation, a silastic catheter was placed in the jugular vein for infusion of solutions. Food was not available during the infusion. Hepatectomized animals were infused either with a solution containing 10% glucose, 143 mM NaCl, 13 mM K_2HPO_4 , 2.3mM $MgSO_4$, and 1.5 mM $ZnCl_2$, pH 7.4 (group A) or the same solution plus 0.24 M branched chain amino acids (0.08 M each of valine, leucine and isoleucine) (group B). The control groups C and D received the glucose solution alone. The solutions were infused at a rate of 0.6 ml/h/100g body weight. Rats were housed in individual cages during infusion and warmed by proximity to a light bulb. Rectal temperature was maintained at 35-37° C.

The rats were decapitated 18 h after hepatectomy or sham operation. Blood from the neck wound was collected into heparinized beakers, and plasma was separated by centrifugation and kept frozen at -72° C until analysis. The brain was removed and dissected into nine regions: hypothalamus, cortex, striatum, hippocampus, diencephalon, mesencephalon, cerebellum and pons-medulla. The dissection was carried out on a plate chilled on ice and the samples were quickly frozen in liquid nitrogen and kept frozen at -72° C until analyzed.

Infusion of branched chain amino acids and ammonium salts in rats with PCA(study VI)

Two separate experiments were performed. In the first experiment, one group of rats was infused with ammonium salts (a mixture of 0.2 mol/l ammonium acetate and 0.2 mol/l ammonium bicarbonate, pH 7.4) and one group of rats was infused with ammonium salts together with branched chain amino acids. The branched chain amino acid solution was a mixture of leucine, isoleucine and valine (0.1 mol/l each).The rate of infusion was 0.9 ml/100 g body weight per hour. The infusion was continued until the rats lapsed into coma and died. Survival time and deterioration of neurological function was followed. In the second experiment one group of rats was infused with ammonium salts only (A) and one group received ammonium salts and branched chain amino acids (B). The neurological function was assessed hourly. Half of the rats in groups A and B were killed when they had minimal neurological impairment (score 14-13; about 3 h infusion time for Group A and about 6 h for Group B). The remaining rats in each group were killed when they had severe

neurological impairment (score <5; about 6 h infusion time for Group A and about 10 h for Group B). The 15-point neurological battery consisted of testing the following reflexes (85), each with an assigned numerical value.

Flexion Reflex: Each foot was pinched with a tissue forceps. Withdrawal of a foot indicated a positive response. With a value of 1 being assigned for each positive response, a total score of 4 was possible.

Grasping reflex: The rat was held by the loose skin of the back and a small metal rod was touched to the surface of both palms at once. If the rat grasped the rod with the fore-paws, a positive grasping reflex was inferred and a value of 1 was scored.

Righting reflex: The rat was placed on its back and a score of 1 was assigned if it immediately righted itself. The rat was then held at the lumbar area and the body was tilted to the left and then to the right. If the rat's head moved in the opposite direction of the body tilt, a value of 1 was scored for each response.

Placement reflex: The rat was slowly moved in the anterior direction toward the edge of a table. If the fore-paws were placed on the table as the rat approached, a value of 1 was assigned for each correctly placed fore-paw (maximum, 2). The rat was placed on the table with the body axis parallel to the edge of the table. If a hindlimb that was pulled down over the table's edge was immediately replaced on the table, a value of 1 was scored for each hindlimb. The rat was suspended by its tail until the vibrissae touched the edge of the table. If the forepaws were extended to touch the table, a score of 1 was assigned.

Corneal reflex: One eye of the rat was lightly touched with a cotton tipped swab. If an eye-blink reflex immediately followed, a score of 1 was assigned.

Head-shaking reflex: Air was blown into the ear using a small tube. If head shaking occurred in response to this stimulus, a score of 1 was given.

Ammonia tolerance tests in patients with liver cirrhosis(VII)

Patients: The study was carried out in seven male cirrhotic patients. They had all portal hypertension and a history of previous bleedings from oesophageal varices. All patients except one had a mesocaval shunt. The diagnosis was based on clinical, laboratory and roentgenologic findings and was always confirmed by a liver biopsy. The patients had had episodes of encephalopathy. Clinical data on the patients are given in table 2.

Table 2. Clinical and Laboratory Data on the Patients.

Pat no	Age yrs	First bleeding	Treatment	Etiology of liver cirrhosis	Albumin * g/l	Prothromb. ^a ** index	ASAT ^b *** μ kat/l	Psychological test
3 hour ammonia test								
1	65	jan 81	sclerotherapy	alcohol	36	50	0.6	normal
2	52	nov 78	shunt ^c jul 78	" "	34	26	0.6	normal
3	56		shunt ^c oct 84	" "	35	45	1.0	slight enc. ^d
4	69	oct 79	shunt ^c feb 80	sarcoidosis	28	50	1.1	slight enc. ^d
5	59	nov 80	shunt ^c dec 80	alcohol	38	45	0.7	pronounced enc. ^d
6 hour ammonia test								
6	65	jan 81	sclerotherapy	alcohol	36	50	0.6	normal
7	41	apr 83	shunt jan 84	" "	38	50	1.0	slight enc. ^d
8	44	apr 84	shunt sept 84	" "	34	>50	0.6	slight enc. ^d

*normal range 36-48g/l; **normal range 70-130 %; ***normal range <0.67 μ kat/l.

Patient number 1 and 6 is the same.

^a Prothromb. = prothrombin; ^b ASAT = aspartate-aminotransferase; ^c shunt = mesocaval interposition shunt; ^d enc. = encephalopathy

Ammonia Tolerance Test: The patients were fasted over night. Tests were started in the morning. Ammonium chloride was dissolved in a glass of water. Five patients received ammonium chloride (2g) at the beginning of the test and another dose after 90 minutes. CSF samples (lumbar) were taken before and 180 minutes after administration of ammonium chloride. Three patients received ammonium chloride (2g) at the beginning of the test and after 90, 180 and 270 minutes, CSF was collected before the start of the test and after 360 minutes. Blood samples were drawn from a peripheral vein before administration of every dose of ammonium chloride and 30 minutes thereafter and at the end of the tests before taking the CSF samples.

Psychometric tests: The patients were regularly tested by a clinical psychologist and the tests were performed within a month preceding the ammonia tolerance test. The psychometric tests utilized in this study are in general use at the Department of Psychiatry, University of Lund, to diagnose diffuse organic brain dysfunction. The test battery was designed to evaluate visual memory and cognitive motor functions. Lowered performance of these tests can reflect a slowing of general response, concentration difficulties and fatigue typical of diffuse brain dysfunctions (86,87,88). The following tests were used:

Visual Retention Test (Benton): Test of conceptual memory, visual perception abilities and memory for geometrical forms.

Perceptual speed (Digit Symbol Test): A visual motor test that tests the capacity to code symbols with digits.

Dot Test (Bourdon Wiersma): This test is very sensitive to concentration difficulties, lack of tenacity and to fatigue.

Colour Word Test: It is used to assess cognitive flexibility.

Manual Dexterity (cylinder board): Tests the capability to combine visual perception and hand motor function.

Trailmaking Test: Tests the cognitive motor abilities.

If a patient in one tested cerebral function deviates more than two standard deviations, he is considered to have slight HE. If he deviates in two functions or more, he is considered to have pronounced HE. The test results are corrected with regard to age and intellectual capacity.

Extractions and analytic procedures

Amino acid assay in experimental animals

Blood: Rats were killed by decapitation and blood from the neck wound was collected into heparinized beakers. In dogs, blood was drawn from a leg vein into heparinized glass tubes. The plasma was separated by centrifugation and kept frozen at -72° C until analysis. Plasma was deproteinized by mixing 0.5 ml of plasma with 1.5 ml of 5% (wt/vol) sulfosalicylic acid, pH 1.7 containing 133 nmol/ml thienylalanine as internal standard. After vortexing, the samples were centrifuged (30,000 x g, 4° C) and passed through a 0.45 micron Millipore filter and analyzed directly.

Cerebrospinal fluid (dogs): The CSF was obtained by cisternal puncture and kept frozen at

-72 °C. The CSF fluid was deproteinized by mixing 0.9 ml CSF with 0.1 ml 37.5 % sulfosalicylic acid, pH 2.0, containing 500 nmol/ml thienylalanine. After centrifugation the samples were passed through a 0.45 micron Millipore filter and analyzed.

Brain: Brain tissue was homogenized in three times its weight of 5% (wt/vol) sulfosalicylic acid, pH 1.4, containing 133 nmole/ml of thienylalanine. Fifty microliters of the supernatant of each brain homogenate was analyzed. To accurately quantitate glutamate, glutamine and aspartate, the supernatant of the brain homogenate was further diluted 1:25 with 0.15 M Li citrate buffer, pH 2.2, and analyzed using a special short analysis program.

Plasma and brain amino acids in experimental animals were determined by a Beckman 121-MB amino acid analyzer with automatic integration.

Amino acid assay in humans

Quantitative determination of amino acids in plasma and in CSF, deproteinized by sulfosalicylic acid, was carried out by a column chromatographic technique using a Biotronik LC 5000 automatic amino acid analyzer with a lithium buffer system.

Ammonia assay in experimental animals

Samples for blood and brain ammonia measurement were obtained in a way designed to minimize postmortem changes of ammonia levels. Under ether anesthesia blood was drawn from the vena cava (II,III,IV,) or abdominal aorta (VI). The blood was transferred to chilled tubes containing heparin. The skull was then exposed by a midline incision and a plastic funnel was positioned over the calvarium. The brain was then frozen in situ by pouring liquid nitrogen into the funnel for 3 minutes, during which time the rats were alive and breathing. The animals were then decapitated and the head was completely frozen by immersion in liquid nitrogen.

Plasma was immediately separated by centrifugation (2000 x g, 15 min, 4 °C) . Plasma was deproteinized by addition to a tube containing an equal volume of frozen 0.5 M HClO₄ to which an additional volume of ice-cold 0.5 M HClO₄ was added, and the samples homogenized with a Polytron homogenizer (Brinkman Instruments). After centrifugation (10,000 x g, 15 min, 4 °C), the supernatant was neutralized to pH 7.0-7.5 by addition of 2.0 M KHC₃O₃; precipitated KClO₄ was removed by centrifugation (10,000 x g, 15min, 4 °C). Brain tissue (0.4-0.7 g) was weighed while frozen and transferred to tubes containing 0.2 ml of frozen 0.5 M HClO₄ . An additional 2.0 ml of ice-cold 5 mM EDTA was added to each

tube and the mixture homogenized for 10 s (on ice). Homogenates were further treated to yield neutralized extracts as described above.

Ammonia was measured using glutamate dehydrogenase in a reaction mixture containing in 1,0 ml 0.25 M Tris buffer, pH 8.0, 2 mM α -ketoglutarate, 0.16 mM NADPH and 4.3 units of glutamate dehydrogenase. Neutralized sample extract was added in amounts (0.1-0.5 ml) depending on expected ammonia content and, when necessary, deionized water was added to bring the final volume to 1.0 ml. The reaction was initiated by addition of sample and the mixture was incubated at room temperature for 60 min, at which time the absorbance at 340 nm was measured. A blank from which enzyme was omitted was prepared for each sample.

Ammonia assay in humans

Ammonia was determined with the glutamate dehydrogenase method using kit from Boehringer Mannheim GmbH.

Assay of neurotransmitters and metabolites in experimental animals

Levels of serotonin (5-HT), 5-hydroxyindole acetic acid (5-HIAA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA)(III), norepinephrine (NE) and tryptophan (paper I) were assayed by high performance liquid chromatography with electrochemical detection, according to a modification of the method of Loullis et al (89). The brain regions were weighed and homogenized (10 x weight) in 1 N formic acid:acetone (that contained N-methyldopamine, NMeDA, as an internal standard in study VI). After centrifugation (10,000 x g, 10 min, 4° C) the samples were divided into two by transferring 2.5 ml of supernatant to each of two tubes containing 7.5 ml heptane:chloroform. The samples were next shaken (10 min) and centrifuged (2,500 x g, 10 min, 4° C) with the organic phase being discarded. In one set of samples, DA and NMeDA were adsorbed onto 100 mg of activated alumina and the pH of the mixture was adjusted to 8.2 - 8.4 with 0.5 M tris buffer containing 2 mM EDTA and 0.03% sodium metabisulfate. The alumina was washed twice with 10 ml distilled deionized water and eluted from the alumina with 0.1 M HCl. Twenty μ l of each of these samples were injected onto the column to separate and quantify NE, NMeDA and DA. The aqueous phase of the other set of samples was dried under a stream of nitrogen, with the samples being reconstituted in 0.8 ml of mobile phase buffer (10% methanol and 90% of 0.1M $\text{KH}_2\text{P}_4\text{O}_4$, 0.185 mM sodium octylsulfate, 0.195 mM EDTA; pH 3.15) and analyzed.

Assay of indoleamines in humans

5-Hydroxytryptophan (5-HTP), serotonin (5-HT), and 5-Hydroxyindoleacetic acid (5-HIAA) were measured in the CSF by high performance liquid chromatography (HPLC) with electrochemical detection.

After addition of perchloric acid (0.4 M), the CSF samples were centrifuged at $34\,000 \times g$ for 10 min, and the supernatant was then filtered. For each analysis of 5-HTP, 5-HT, and 5-HIAA performed in the same run, 0.5 ml of this filtrate was placed in the automatic injector after addition of 100 ng alfa-methyl 5-HT as an internal standard. 10 μ l 5% L-cysteine solution was added to the filtrate as an antioxidant for 5-HIAA.

Glucose assay in humans:

Glucose was determined by the glucose oxidase technique.

Insulin and glucagon assay in humans:

The insulin and glucagon concentrations were measured with radioimmunoassay according to Heding (90) and von Schenck (91) respectively.

Plasma competitor ratios (study I)

One aim of this study was to determine whether the rise in the concentrations of various LNAA in the central nervous system could be explained solely by changes in circulating LNAA concentrations.

Studies in rats have demonstrated that the LNAA compete for a common blood-brain transport system (52, 61). Each LNAA has a characteristic affinity for the transport system. The effects of competition among plasma amino acids on cerebrospinal fluid levels of LNAA have been demonstrated in dogs after PCA (37). Although no blood-brain transport K_m (affinity) values are available for normal dogs or dogs after PCA, studies in dogs have suggested that the rank-order of transport K_m 's is similar to that in rats (92).

The availability of a particular amino acid for blood-brain transport (plasma competitor ratios) was expressed in a way which accounted for the competition among plasma amino acids due both to their different concentrations in plasma and to their presumed relative affinities. The plasma competitor ratios (PCR) of the studied amino acids were calculated in a way similar to that used by Fernstrom and Faller (54) for calculating the brain influx rate with the modification that the V_{max} of transport was considered to be 100% (93).

One interpretation of uptake, expressed in this fashion, is that the calculated values represent the fraction of maximum transport activity expended on a particular amino acid. Making these calculations, we assumed that blood-brain LNAA transport does not greatly differ in dogs and rats and that PCA does not alter the affinities of the amino acids for the transport system.

The PCR were calculated as follows:

$$PCR_x = \frac{AA_x}{[Km_x \cdot (1 + AA/Km) + AA_x]} \cdot V_{max}$$

where AA_x is the plasma concentration of the NAA_x; Km_x is the transport Km of AA_x; Km is the transport Km of competing NAA; AA is the plasma concentrations of competing NAA; and V_{max} is the transport V_{max} = 100%. The Km's of transport for each neutral amino acid from Pardridge and Mietus (61) were as follows: threonine 730 nmol/ml (94); valine 510 nmol/ml; isoleucine 250 nmol/ml; leucine 100 nmol/ml; methionine 180 nmol/ml; tyrosine 150 nmol/ml; phenylalanine 110 nmol/ml; and histidine 240 nmol/ml. Tryptophan concentrations were not included in the calculation since the actual amount of non-albumin bound tryptophan was not determined. This fraction of the plasma tryptophan is relatively small and therefore exerts only slight competitive effect.

Calculation of blood-brain transport of phenylalanine and leucine (study IV)

Carotid Injection Technique

The brain uptake index (BUI) was measured by the method of Oldendorf (95). Under sodium pentobarbital anesthesia (45-50 mg/kg) 15 sec after a rapid injection into the carotid artery of 0.2 ml of buffered Ringer's solution (pH 7.4, 10 mM HEPES) containing ¹⁴C-butanol, ¹⁴C-amino acid or ¹⁴C-sucrose (1-2 µCi/ml) and [³H]-water (5-10 µCi/ml) the rats were decapitated. A piece of frontal cortex (0.15 - 0.25 g) was dissected from the side of brain ipsilateral to the injection and solubilized in tightly-closed tubes with 1.0 ml TS-1 tissue solubilizer (Research Products International, Mount Prospect, IL) at 50 °C for 3-4 h. Samples were counted in an LKB Model 1217 liquid scintillation counter using external standardization and dual label correction.

The brain uptake was calculated as:

$$\text{BUI} = \frac{{}^{14}\text{C}/{}^3\text{H} \text{ (dpm in brain)}}{{}^{14}\text{C}/{}^3\text{H} \text{ (dpm in injection solution)}} \times 100$$

This method permits measuring the brain uptake of a single substance without interference of other substances competing for the same transport system.

Calculations of transport parameters

The rate of cerebral blood flow (F; measured in $\text{ml min}^{-1} \cdot \text{g}^{-1}$) and extraction of water relative to butanol (E_{HOH}) were determined as described by Pardridge et al (96) with the difference that we followed the butanol washout at 0.25, 1.0, and 2.0 min after carotid injection of [${}^{14}\text{C}$]-butanol and [${}^3\text{H}$]-water, omitting the four-minute point to avoid possible error due to recirculation. Since the treatments involved may have influenced the cerebral blood flow, this parameter was determined separately for each treatment group. The F was calculated as follows:

$$F = \frac{K_b \cdot V_1}{E_b}$$

where K_b = the rate constant (min^{-1}) of [${}^{14}\text{C}$]-butanol efflux from brain calculated from a linear regression of butanol washout data during a 2 minute circulation period after carotid injection, V_1 = the ratio (0.88ml/g) of butanol distribution in brain to blood, and E_b = the extraction of butanol, assumed to be 1.0. The E_{HOH} was calculated as follows:

$$E_{\text{HOH}} = [\text{BUI}_{\text{HOH}}(15 \text{ sec})] e^{-K_b t}$$

where $\text{BUI}_{\text{HOH}}(15 \text{ sec})$ = the uptake index of [${}^3\text{H}$]-water relative to [${}^{14}\text{C}$]-butanol at 15 sec after injection and t = the mean time (about 10 s) between clearance of the bolus through the head and decapitation.

The extraction fraction of the unidirectional influx (E; measured in %) for the ^{14}C -labeled amino acid was calculated as follows :

$$E = (\text{BUI}_{\text{amino acid}} - \text{BUI}_{\text{sucrose}}) (E_{\text{HOH}}) / 100$$

$\text{BUI}_{\text{amino acid}}$ and $\text{BUI}_{\text{sucrose}}$ are the uptake indices relative to $[^3\text{H}]$ -water of amino acid or sucrose, respectively. Subtraction of $\text{BUI}_{\text{sucrose}}$ provided a correction for residual radioactivity remaining in the brain vasculature. On the basis of E and F the unidirectional clearance (V/C ; measured in $\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$) was calculated as follows:

$$\frac{V}{C} = E F$$

where C = the mean capillary concentration of amino acid; V = unidirectional influx .

The V/C data calculated from the different BUI data were fitted to the equation below, and the K_m and the V_{max} of the saturable component of transport, and the K_d or constant of non-saturable transport, were determined from a nonlinear regression analysis as described by Pardridge et al (96). It was assumed that the saturable uptake (V_{max} , maximal velocity, and K_m , the affinity) followed Michaelis-Menten kinetics and that the non-saturable component (K_d) was linear with respect to amino acid concentration.

$$\frac{V}{C} = \frac{V_{\text{max}}}{(K_m + C)} + K_d$$

The mean capillary concentration, C , was calculated from the arterial injection solution concentration (C_a) as follows:

$$C = \{E / -\ln(1-E)\} \cdot C_a$$

High values of E, as observed in ammonia infused rats, required correction for change in concentration due to uptake during passage through the capillary as described by Pardridge and Mietus (61).

Electron microscopy studies of the integrity of the blood-brain barrier

Rats were injected with horseradish peroxidase (HRP) as described by Laursen and Westergaard (59). Areas from the cerebral cortex, cerebellum, basal ganglia (corpus striatum) and brain stem were dissected out and examined. The electron microscope used was a JEOL-JEM Model 100c.

Statistics

Study I: Statistical analysis of differences among serial samples of plasma and CSF was determined by analysis of variances and Newman-Keuls test. Correlation was tested by Pearson product-moment correlation. Comparison between amino acid concentrations in brain regions of control dogs and dogs killed in various stages of encephalopathy is by the non-parametric Kruskal-Wallis test.

Study II, III: Significant differences between treatment groups were calculated by analysis of variance followed by Newman-Keuls test.

Study IV: Determination of significance of differences between treatment groups in the kinetic parameters themselves proceeds in a two-step process (97) First, the overall significance of difference between two regression curves is assessed by calculation of an F-statistic defined as follows:

$$F_{3,6} = \frac{\frac{SS_c - (SS_1 + SS_2)}{3}}{\frac{SS_1 + SS_2}{6}}$$

where $F_{3,6}$ = the F-statistic for $df_1 = 3$ and $df_2 = 6$ (3 being the total number of unknown parameters solved for in each group and 6 being the total number of unknown parameters in both groups combined), SS_c = error sum of squares of the combined data of both test groups (=residual mean square error x the number of data points minus 3) and SS_1 and SS_2 are the error sum of squares of each group taken alone. Only if an overall significant F was

obtained, was the significance of differences, in particular parameters, tested.

To determine statistical significance of a difference in a particular parameter, a t-statistic is calculated as follows:

$$t = \frac{P_1 - P_2}{\sqrt{(SD_1)^2 + (SD_2)^2}}$$

where P_1, P_2 = the value of the particular parameter (K_m , etc.) determined and SD_1, SD_2 = the Standard Deviation for that parameter as calculated by the nonlinear regression program. The degrees of freedom appropriate for the t-table is $(N_1 + N_2) - 2$ which, in most cases, was between the $df = 60$ and $df = 80$ lines on the table.

Study V: Multivariate analysis of variance was performed on amines and amino acids to determine differences among the treatment groups. Subsequently Duncan's test was used for comparison between treatment groups.

Study VI: Significance of differences between groups was determined by two-way analysis of variance followed by Scheffe's t-test (ammonia data) or by Student's t-test (amino acid and neurotransmitter data).

Study VII: Correlation was tested by Pearson product-moment correlation.

BLOOD BRAIN TRANSPORT OF LARGE NEUTRAL AMINO ACIDS (I,II, III, IV,VII)

Increased blood-brain transport of LNAA has been described in experimental models of liver disease (31,56) and in patients with liver cirrhosis (57). The mechanism underlying the deranged blood-brain transport of LNAA in liver disease is not clarified. Liver failure is associated with hyperammonemia, altered LNAA pattern in plasma, and high levels of several of the LNAA acids in brain. It is unclear what role alterations in the amino acid levels in plasma and brain play in the increased blood-brain transport of LNAA. It is also unclear what importance hyperammonemia itself and the morphological changes in the blood-brain barrier have for the transport of amino acids across the blood-brain barrier.

The role of plasma and brain amino acid changes has been predominantly studied in rats with PCA and these data suggest that elevated brain concentrations of glutamine, the end product of ammonia detoxification in brain, stimulate the LNAA transport across the blood-brain barrier (60,62). We studied the amino acid changes in plasma, in cerebrospinal fluid (CSF), and in brain in dogs before and after PCA to see if similar changes are present in other species (Study I). After PCA there was an increase in the CSF concentrations of glutamine, tyrosine, tryptophan, methionine and phenylalanine. The increase of these amino acids in CSF exceeded the increase in their absolute plasma concentrations and the calculated competitor ratios (PCR). For all CSF amino acids except threonine, the correlation to CSF glutamine was better than to the PCR. The brain concentrations of phenylalanine and tyrosine correlated equally well both to the PCR and to the brain glutamine concentrations, whereas the brain concentrations of methionine and histidine correlated better to the brain glutamine concentrations than to the PCR. This may indicate that in the central nervous system (CNS) the LNAA concentrations are more influenced by the glutamine concentrations in CNS than by the LNAA concentrations in plasma.

The discrepancy between the plasma and the CNS concentrations of the LNAA after PCA in dogs could be explained by an increased uptake of LNAA into brain. This has been demonstrated in rats after PCA (31,56), in humans with liver cirrhosis (57), but not in dogs after PCA (98). The differences among the brain concentrations of the LNAA may be accounted for by differences in affinity to the LNAA transport system. Some amino acids, such as phenylalanine, tyrosine, leucine and histidine, have high affinity to the transport system, whereas others, such as threonine and valine, have low affinity to it (94).

The relatively rapid metabolism of leucine in the brain tissue (99) and its relatively unchanged plasma levels may explain that the concentrations of leucine in brain did not change much after PCA in spite of its high affinity to the LNAA transport system. Since the LNAA, except glutamine and tyrosine, cannot be synthesized in the brain, their brain concentrations are, to some extent, influenced by their rate of transport across the blood-brain barrier. Glutamine has a low affinity to the LNAA transport system, and the sharp increase in the glutamine concentrations in brain after PCA is probably a consequence of increased ammonia detoxification (13). High brain concentrations of glutamine may, by competition for the same carrier system, inhibit the efflux of other LNAA out of brain (60,62). This together with the altered plasma competitor ratios could explain the observed changes in brain LNAA concentrations after PCA. Other investigators argue that glutamine is a weak competitor for the LNAA blood-brain transport system and, consequently, it would be unlikely for glutamine to exert such an influence. On the other hand, if glutamine is accumulated to very high concentrations in the endothelial cells, the presumed anatomical location of the blood-brain barrier, these high concentrations of glutamine would be able to compete for transport out of brain with the other LNAA. For further studies of the mechanism of the LNAA transport into brain we investigated the effects of inhibiting the glutamine synthesis by methionine sulfoximine (MSO) on plasma and brain amino acids in rats after PCA (Study II). We also studied the effects on the LNAA concentrations in plasma and brain, and on the kinetic parameters of the blood-brain transport of phenylalanine and leucine in normal rats infused with ammonium salts with and without pretreatment of MSO (Study III and IV). The next step was to investigate the relationship between the concentrations of glutamine and the other LNAA in CSF in patients with liver cirrhosis before and after administration of ammonium salts (Study VII).

Pretreatment with MSO in shunted rats increased the plasma levels of BCAA, threonine, alanine, lysine and methionine, and decreased the plasma levels of glutamine compared to untreated shunted rats, whereas MSO treatment did not change the plasma levels of tyrosine, phenylalanine and histidine (Fig 2).

Administration of MSO decreased the brain levels of threonine, valine, methionine, leucine, and especially of tyrosine, phenylalanine, histidine (Fig 3) and glutamine, whereas the levels of alanine and lysine increased, compared to non-pretreated shunted rats.

Infusion of ammonium salts in normal rats (Study III) had a very slight effect on the plasma concentrations of LNAA (Fig 4), while the brain concentrations of LNAA increased (Fig 5). MSO treatment had no effect on the plasma amino acids except for a decrease in phenylalanine and, especially, in glutamine. Pretreatment with MSO sharply reduced the

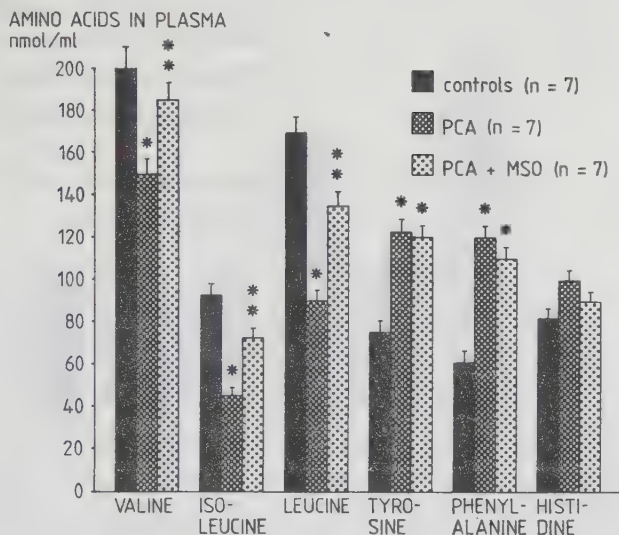


Figure 2. Plasma amino acid concentrations in rats after PCA with and without MSO treatment. * = $p < 0.01$ compared to controls; ** = $p < 0.01$ compared to PCA without MSO treatment.

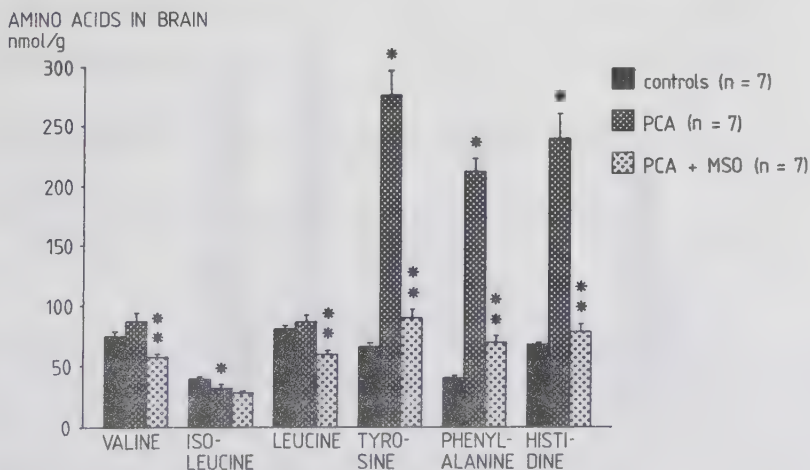


Figure 3. Brain amino acid concentrations in rats after PCA with and without MSO treatment. * = $p < 0.01$ compared to controls; ** = $p < 0.01$ compared to PCA without MSO treatment.

AMINO ACIDS IN PLASMA nmol/ml

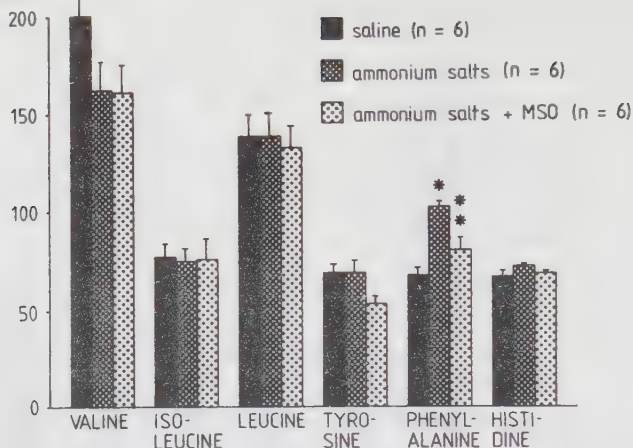


Figure 4. Plasma amino acid concentrations in rats after infusion of ammonium salts with and without MSO treatment. * = $p < 0.05$ compared to saline infusion; ** = $p < 0.05$ compared to infusion of ammonium salts without MSO treatment.

AMINO ACIDS IN BRAIN nmol/ml

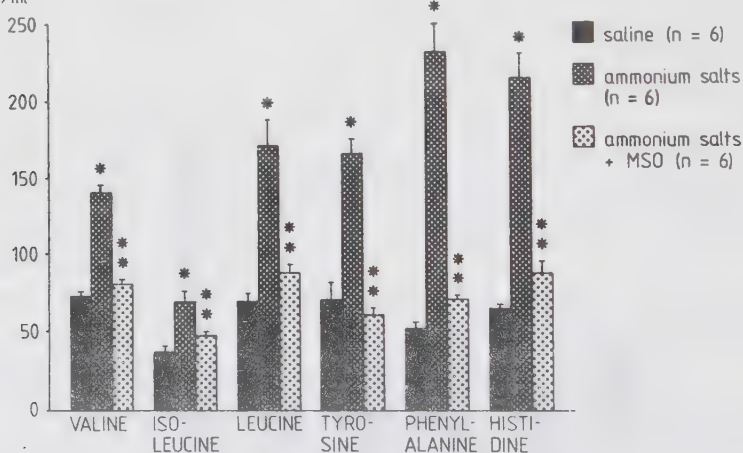


Figure 5. Brain amino acid concentrations in rats after infusion of ammonium salts with and without MSO treatment. * = $p < 0.05$ compared to saline infusion; ** = $p < 0.05$ compared to infusion of ammonium salts without MSO treatment.

Ammonia infusion increased the K_d and V_{max} of phenylalanine transport about 170% and 80%, respectively, compared to controls, while the K_m and V_{max} of leucine transport were increased, respectively, about 100% and 200% (Study IV)(Table 3). Administration of MSO before infusion of ammonium salts inhibited these changes in the transport across the blood brain barrier.

Table 3
Effects of Ammonia and MSO on the Kinetics of Blood-Brain Transport
of Phenylalanine and Leucine into Brain Cortex

<u>Phenylalanine</u>	K_m	V_{max}	K_d
Treatment:	(mM)	(nmol·min ⁻¹ ·g ⁻¹)	(ml·min ⁻¹ ·g ⁻¹)
None	0.09 ± 0.01	21 ± 3	0.033 ± 0.005
NH ₄ ⁺ salts	0.12 ± 0.02	38 ± 6 ^{a,b}	0.090 ± 0.007 ^{a,b}
MSO, NH ₄ ⁺ salts	0.10 ± 0.02	21 ± 3	0.031 ± 0.006
<u>Leucine</u>			
None	0.20 ± 0.03	39 ± 4	0.013 ± 0.003
NH ₄ ⁺ salts	0.40 ± 0.08 ^{a,b}	108 ± 23 ^{a,b}	0.021 ± 0.012
MSO, NH ₄ ⁺ salts	0.11 ± 0.02	20 ± 3	0.008 ± 0.005

^a Differs significantly from the same parameter in untreated control rats;
 $p < 0.05$ at least.

^b Differs significantly from the same parameter in MSO-pretreated rats;
 $p < 0.05$ at least.

MSO treatment resulted in a sharp increase in the brain concentrations of ammonia. The results from study II,III,IV demonstrated that alterations in the plasma concentrations of the LNAA are of minor importance for increased LNAA concentrations in brain after PCA and after infusion of ammonium salts in rats. The changes in the brain concentrations of the LNAA were dependent on an intact glutamine synthesis and were not a direct effect of

ammonia itself. Contrary to this, Mans et al (65) showed that the rise in the glutamine concentrations after infusion of ammonium salts came much earlier than the rise in the brain uptake of tryptophan. They also showed that after the end of the ammonia infusion, normalization of the brain uptake of tryptophan came before a normalization of the brain concentrations of glutamine. They concluded that the changes in the blood-brain transport of the LNAA in conditions with hyperammonemia were due to a direct effect of ammonia and not to an effect of glutamine. If it takes some time for high glutamine concentrations to induce changes in the blood-brain transport, this could be one explanation of the different results. It is also our experience that in normal rats the glutamine concentrations in brain after ammonia infusion must be much higher than normal to induce changes in the blood brain transport. This could explain the finding of Mans et al that the brain uptake index of tryptophan was normalized before the brain concentrations of glutamine.

Ammonia tolerance tests performed in humans with liver cirrhosis and a history of encephalopathy (Study VII) resulted in a marked increase in glutamine and LNAA concentrations in CSF in only one out of 7 patients, despite high CSF ammonia levels. This patient was the only one with pronounced encephalopathy. The different effects of ammonia administration on humans compared to the effects on rats could be explained by different length of exposure to ammonia, too good liver function in patients studied, or a species difference in reaction to ammonia. The good correlation, however, between the CSF glutamine concentrations and the CSF concentrations of phenylalanine ($r=0.90$), tyrosine($r=0.90$), histidine($r=0.90$), and methionine ($r=0.92$) may support the concept that the CSF glutamine concentrations play a role in the blood-brain transport of LNAA also in humans (Fig 6).

In order to evaluate the data from the experimental studies one has to consider a relationship between increased blood-brain transport of LNAA and structural changes in the blood-brain barrier after infusion of ammonium salts and after PCA. Swelling of the astrocytes, surrounding the cerebral capillaries, has been reported in humans with liver coma and in experimental animals after hepatectomy, PCA, and ammonia infusion (21,59). This may cause a disruption of the physical integrity of the blood-brain barrier and an increased permeability not only for LNAA but also for inulin and sucrose, to which the blood-brain barrier is normally virtually impermeable. In rats, intact basement membrane and intact tight junctions between the cells have been found four weeks after PCA (59). The present study has demonstrated that ammonia infusion resulted in a watery swelling of the astrocytes already within 24 hours and that this swelling did not seem to be affected by MSO treatment.

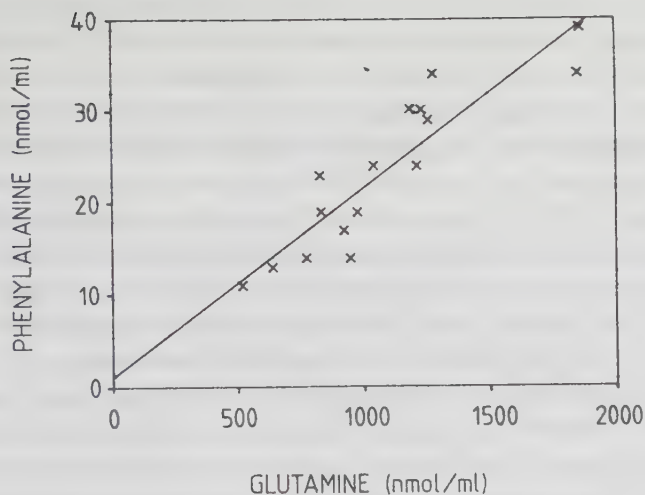


Figure 6. The relationship between the CSF concentrations of glutamine and the CSF concentrations of phenylalanine in patient with liver cirrhosis before and after ammonia tolerance test. Correlation coefficient = 0.90.

The interendothelial tight junctions were intact and a normal basement membrane was maintained around the cerebral capillaries. Since MSO normalized the transport kinetics of phenylalanine and leucine, the swelling of the astrocytes does not seem to be related to the altered blood brain transport of these amino acids.

The BUI of sucrose, which is normally not taken up into brain, was higher in ammonia-infused rats than in normal rats. As no disruption of the integrity of the blood-brain barrier was discovered, reduced blood flow through some capillaries, due to increased swelling of the astrocytes, may prolong the washout of sucrose in some capillaries without affecting the total blood flow of the brain.

The uptake of tyrosine and leucine *in vitro* by isolated brain microvessels can be stimulated by preloading the microvessels with either glutamine or another LNAA, methionine. This stimulation of uptake declines rapidly after transfer of the microvessels to an amino-acid-free medium in parallel with the efflux of the preloaded amino acids from the microvessels (67,100,101). The rise in transport V_{max} after ammonia infusion could be the result, rather than the cause, of high brain LNAA concentrations. If so, the increase in

brain LNAA after ammonia infusion must be explained by some factor other than an increased influx. This factor could be either a decreased rate of efflux of LNAA from brain, or an increase in free amino acids in brain due to accelerated proteolysis, or to reduced protein synthesis. We support the former of these two explanations, since if ammonia disrupted protein metabolism in brain, it would be difficult to explain how MSO could block that disruption. Another mechanism could be that high brain levels of glutamine may stimulate synthesis of LNAA via transamination of their alfa-keto acids, forming LNAA and alfa-ketoglutaramate (16) and thereby increase the LNAA levels in brain. Elevated brain levels of alfa-ketoglutaramate have been found in the CSF of patients with hepatic coma (16). However, the brain levels of both threonine and histidine were elevated by ammonia infusion and these amino acids are not transaminated to alfa-ketoglutaramate.

The LNAA share the same transport system across the blood-brain barrier (52). Since glutamine is transported by the LNAA transport system, high brain glutamine concentrations, secondary to ammonia detoxification, may inhibit the efflux of other LNAA from brain (60). The present results are in agreement with the hypothesis that MSO prevents the rise in transport V_{max} by inhibiting brain glutamine synthesis and thereby the accumulation of glutamine and other LNAA in brain after ammonia infusion.

In this study (IV), ammonia infusion significantly increased the transport K_d of phenylalanine but not that of leucine, and the transport K_m of leucine but not that of phenylalanine. Other investigators have reported an increase in V_{max} and K_m - but not in K_d - for leucine in isolated bovine microvessels in the presence of high levels of ammonia, similar to what is found in this study (IV) (100). After PCA in rats, higher clearance by brain of circulating phenylalanine and leucine has been demonstrated (102). The results from these studies are in agreement with the findings in the present study. The reasons for the different effects of the ammonia infusion on the K_d and the K_m of leucine and phenylalanine remain unclear. These effects do not seem to be related to ammonia itself, since the transport kinetics were normalized by MSO and the brain ammonia concentrations were simultaneously increased.

The mechanism of the increased blood-brain transport of LNAA remains unknown. Two hypotheses have been proposed and in both of them exchange transport of glutamine plays an important role. One suggests involvement of the gamma-glutamyl cycle (103,104). Gamma-glutamyl-glutamine (provided from the gamma-glutamyl-cycle) reacts with a LNAA and provides gamma-glutamyl-LNAA and glutamine. Glutamine is left in the capillary lumen, while gamma-glutamyl-LNAA is transported into the cell where the LNAA is split off and gamma-glutamyl-glutamine is formed again. MSO inhibits both glutamine synthetase

and gamma-glutamylcysteine synthetase, which are enzymes involved in the gamma-glutamyl cycle. If the gamma-glutamyl cycle is involved in the blood-brain transport of LNAA, the stimulating effect of ammonia on the blood brain transport may imply not only stimulation of the glutamine synthesis but also an unknown stimulating effect on the gamma-glutamyl cycle. In isolated brain microvessels MSO did not modify the neutral amino acid uptake when ammonia was absent, which does not support the "gamma-glutamyl hypothesis" (100).

The other hypothesis suggests involvement of a mobile carrier mechanism (60). If the LNAA are transported across the blood brain barrier by a "mobile carrier" mechanism, high concentrations of glutamine and other LNAA within the capillary endothelial cells may accelerate the rate of the carrier transport through the blood-brain barrier. By reducing the glutamine concentrations in brain, MSO may prevent accelerated exchange transport and thereby a rise in V_{max} after infusion of ammonium salts.

The results from study II,III and IV strongly suggest that hyperammonemia in rats is involved in the transport of LNAA across the blood-brain barrier through the glutamine synthesis. The present studies do not answer the question whether the rise in the brain levels of the LNAA follows or precedes the changes in blood-brain transport.

It remains to be shown whether hyperammonemia in patients with liver cirrhosis increases the blood-brain transport of the LNAA.

ADMINISTRATION OF BRANCHED CHAIN AMINO ACIDS (V,VI)

After hepatectomy and glucose infusion, the plasma concentrations of all amino acids increased except for glutamic acid and BCAA. Addition of BCAA to the infusion solution resulted in higher plasma concentrations for serine, BCAA, glutamic acid, glutamine, alanine and glycine, while there was a decrease in the plasma concentrations of tryptophan, lysine, histidine, methionine, threonine, phenylalanine and tyrosine .

Reduced plasma concentrations of the other LNAA as a result of BCAA treatment have been reported by other investigators in humans (3,23) and in experimental animals (32,37), and could be interpreted as a direct effect of the BCAA on the protein metabolism in peripheral tissues (76).

Following hepatectomy and glucose infusion, the brain concentrations of all amino acids rose except the BCAA, glutamic acid and serine. After addition of BCAA to the infusion solution there was a decrease in the brain concentrations of tryptophan, histidine, lysine, methionine, threonine, phenylalanine, and tyrosine in all brain regions, whereas the brain concentrations of alanine and glutamine increased in some brain regions .

There were regional differences in the brain concentrations of the amino acids and indoleamines, but the response to the different treatments was similar in all regions.

After BCAA infusion, the decrease in the brain concentrations of phenylalanine, methionine, tyrosine, threonine, histidine and tryptophan in all regions seemed to be greater than could be expected from the changes in their plasma concentrations. Also in shunted rats, BCAA infusion compared to ammonia infusion alone (Study VI) showed the same result. This effect of BCAA treatment is probably a result of competition for the same transport system and suggests that, with regard to competition, the blood-brain barrier seems to keep its integrity even under severe metabolic disturbances. Similar observation has been reported by other investigators after hepatectomy (32) and after ammonia and BCAA infusion (63).

Hepatectomy and glucose infusion resulted in a great increase in the tryptophan concentrations in the different brain regions. Compared to shunted rats, the concentrations of 5-hydroxyindoleacetic acid (5-HIAA) increased significantly in all regions, except for diencephalon. It was only in hypothalamus that hepatectomy gave higher concentrations of serotonin than in shunted rats. In all brain regions, infusion of BCAA in hepatectomized rats resulted in lower concentrations of tryptophan, serotonin and

5-HIAA than in the two control groups. These observations clearly demonstrate the capability of the BCAA to depress the indoleamine concentrations in brain, probably by reducing the influx of tryptophan into brain through competition for transport. No difference in survival could be found after hepatectomy whether the rats were infused with glucose alone or with glucose and BCAA. This showed that the capability of the BCAA to reduce the brain concentrations of LNAAs and indoleamines was of no importance for the survival in this extreme model of liver insufficiency. Similar results after BCAA treatment have been reported in other experimental models with fulminant hepatic failure after CCl_4 injection (105) and after hepatectomy (32). Contrary to these findings, infusion of BCAA and ammonium salts, compared to infusion of ammonium salts alone, prolonged survival and slowed down the deterioration of the neurological functions in rats with PCA (Study VI)(Fig 7).

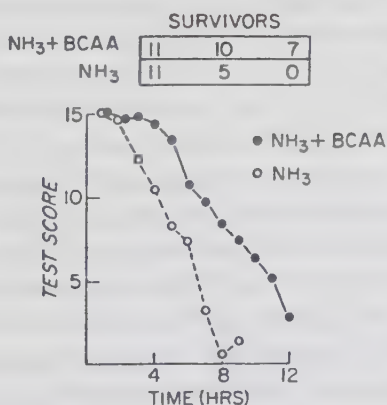


Figure 7. Effect of infusing ammonium salts alone and of infusing ammonium salts combined with BCAA on neurological function and on survival. Neurological function is expressed as test score hourly. Survival is given after 4, 8 and 12 hours of infusion.

Coinfusion of BCAA and ammonium salts demonstrated that the ammonia concentrations were not higher in brain and plasma after 10 hours infusion than after 6 hours, which was in contrast to rats receiving ammonia alone. In the latter group, the ammonia concentrations rose in proportion to the duration of infusion. Moreover, the ammonia concentration both in brain and in plasma was lower after BCAA infusion. These results demonstrate that addition of BCAA to ammonium salts not only prolong survival but also delay neurological deterioration and accelerate the detoxification of ammonia. Although one should take great precaution when applying experimental work to humans, this finding suggests that patients with chronic liver failure exposed to an acute nitrogen load, such as after a gastrointestinal bleeding, could be helped by administration of BCAA to accelerate the ammonia detoxification. Further clinical trials are needed to clarify whether BCAA treatment can accelerate the ammonia detoxification in humans.

BCAA coinfused with ammonia in shunted rats increased the plasma concentrations of glutamine and alanine compared to infusion of ammonia alone. Also in the hepatectomized rats the plasma concentrations of alanine and glutamine increased after BCAA infusion. The BCAA have been reported to stimulate alanine and glutamine syntheses both in vitro and in vivo (80,106,107,108). Glutamine synthesis consumes both glutamic acid and ammonia and may be of importance for ammonia detoxification in liver insufficiency (5). The increase in glutamine and alanine concentrations after BCAA infusion is probably a result of stimulated transamination of BCAA with alpha-ketoglutarate, a process which makes more glutamic acid available for glutamine synthesis. The rise in the alanine concentrations could be due to enhanced glutamate-pyruvate transamination, or to synthesis of alanine from glutamine by the gut (109). The rise in the plasma concentrations of glutamine and alanine after BCAA infusion may be consistent with the assumption that BCAA stimulate the ammonia detoxification (79,80). In agreement with this assumption, inhibition of the glutamine synthesis in rats with PCA (Study II) resulted in an increase in the plasma concentrations of alanine and BCAA. Also in patients with liver cirrhosis, ammonia tolerance tests resulted in a reduction in the plasma BCAA concentrations in most patients (Study VII). On the other hand, 24 hours ammonia infusion with or without inhibition of the glutamine synthesis in normal rats (Study III) did not result in a decrease in the plasma BCAA concentrations. After PCA in rats, it takes a few days before there is a decrease in the plasma concentrations in BCAA (55). It might be necessary to extend the infusion time to more than 24 hours to demonstrate that ammonia infusion reduces the plasma concentrations of BCAA in healthy animals.

Increased brain concentrations of 3,4-hydroxyphenylacetic acid (DOPAC), the metabolite

of dopamine (DA), were observed in both treatment groups, whereas the brain concentrations of norepinephrine (NE) decreased as neurological function declined. One explanation of prolonged survival after infusion of BCAA into rats with portacaval anastomosis could be that the BCAA delayed the decline of the NE concentrations, perhaps as a result of lower ammonia concentrations in brain. A decrease in the NE concentrations in brain has been observed after hepatectomy and liver necrosis in rats (32,40). Also in humans with hepatic coma, determination of the catecholamines in CSF suggested an inhibition of the synthesis of NE from DA (41). Whether this inhibition of the norepinephrine synthesis following coma is a cause or an effect of the coma cannot be decided. No consistent relationship could be found between the neurologic function and the brain levels of serotonin and 5-HIAA in this model of hyperammonemia. According to the neurotransmitter hypothesis for HE, increased concentrations of serotonin or 5-HIAA should have been expected as the neurological score decreased. One explanation could be that in this model with extreme hyperammonemia -at least three times higher than in shunted rats- the hyperammonemia itself was of major importance for mental deterioration. Another explanation could be that measurements of the indoleamine concentrations do not give any information of the rate of the indoleamine metabolism.

SUMMARY

The etiology of hepatic encephalopathy is unknown and several etiologic factors have been suggested. Ammonia is the oldest one but the correlation between the ammonia concentrations in plasma and the grade of hepatic encephalopathy has been poor, and the mechanism whereby ammonia affects the central nervous system is unclear. Disturbances in the amino acid and neurotransmitter metabolism have been suggested to contribute to hepatic encephalopathy. In liver failure, the accumulation of LNAA in the central nervous system is greater than can be predicted by the changes in their plasma concentrations. Some of these amino acids are precursors of neurotransmitters and high concentrations of them in the central nervous system may contribute to development of hepatic encephalopathy. The mechanism underlying this accumulation of LNAA in the central nervous system is unclear. It has been proposed that high concentrations of glutamine in the central nervous system, secondary to hyperammonemia seen in liver failure, may accelerate the transport of other LNAA into brain and thereby also increase the brain LNAA concentrations.

Although the concentrations of the LNAA in the central nervous system in dogs after portacaval anastomosis somewhat correlated with the LNAA competitor ratios in plasma, better correlations were found with the glutamine concentrations in the central nervous system. These findings suggest that in liver failure glutamine is more important for the increase in the concentrations of the LNAA in the central nervous system than the changes in their plasma concentrations.

After PCA in rats and after infusion of ammonium salts in normal rats there was a rise in the brain concentrations of LNAA. This rise was largely prevented when the glutamine synthesis was inhibited by methionine sulfoximine. Simultaneously, the ammonia concentrations in brain increased. These observations demonstrate that the rise of the LNAA in brain is dependent on an intact glutamine synthesis and is not a result of high ammonia concentrations in itself.

Infusion of ammonium salts in normal rats resulted in an increase in the V_{max} of blood-brain transport of the LNAA phenylalanine and leucine. This effect was completely prevented when the glutamine synthesis was inhibited. The physical integrity of the blood brain barrier was examined by electron microscopy. No disruption was found after infusion of ammonium salts.

Since glutamine and the other LNAA share the same transport system across the blood-brain barrier, high brain concentrations of glutamine may by competition inhibit the efflux of other LNAA out of brain. The result would be an increase in the LNAA in brain. If the LNAA transport is mediated by a "mobile carrier" mechanism, high concentrations of LNAA in brain may increase the rate of the carrier return and enhance the availability of carriers at the luminal surface. The result would be a rise in V_{max} and this may be interpreted as enhanced mobility of an occupied carrier, compared to an unoccupied one. Inhibition of the glutamine synthesis prevented an increase in glutamine and of other LNAA in brain and thus also prevented an accelerated carrier transport and a change in V_{max} .

These observations in experimental animals support the hypothesis that hyperammonemia in liver failure influences the brain concentrations of the LNAA and thereby also the neurotransmission via the glutamine synthesis.

Contrary to this, ammonia tolerance tests in patients with liver cirrhosis, with slight or no HE, did not increase the CSF concentrations of glutamine and the other LNAA despite high CSF ammonia levels. There was a marked increase in glutamine and the other LNAA in CSF after the ammonia load in only one patient with pronounced encephalopathy. A good correlation, however, was found between the CSF glutamine concentrations and the CSF concentrations of phenylalanine, tyrosine, histidine and methionine, suggesting that also in humans glutamine may play a role in the blood-brain transport of LNAA.

Treatment with BCAA in hepatic encephalopathy has been suggested to reduce the concentrations of LNAA in brain and thereby also affect the neurotransmission. It has also been suggested that BCAA could increase the ammonia detoxification in the peripheral tissues and thus protect against hyperammonemia.

Infusion of BCAA into rats 18 hours after hepatectomy demonstrated that BCAA in fulminant hepatic failure reduced the brain concentrations of the other LNAA, except those of glutamine, and also reduced the indoleamine concentrations in brain. In this extreme model of liver failure, however, infusion of BCAA did not improve survival.

In rats with PCA, infusion of ammonium salts with addition of BCAA prolonged life, postponed mental deterioration, reduced the concentrations of ammonia in plasma and in brain, compared to infusion of ammonium salts alone. The plasma glutamine concentrations were higher in rats receiving BCAA. These findings suggest that BCAA can protect against hyperammonemia in chronic liver failure by stimulating the peripheral detoxification of ammonia.

The present work demonstrates an interaction between ammonia and the metabolism of BCAA and an interaction between ammonia and altered permeability of the blood-brain barrier in experimental animals. Ammonia -the old villain of HE - seems to serve as a link between some of the metabolic disturbances in liver failure. Further studies are needed to clarify whether similar changes of potentially pathogenetic substances are observed in man and correlate with the degree of encephalopathy.

FUTURE ASPECTS

The nature of blood-brain transport of LNAA still remains unclear. The results obtained in this study support the concept that the blood-brain transport of LNAA is mediated by a concentration dependent mobile carrier mechanism. Infusion of high concentrations of LNAA would then increase the brain uptake of LNAA. Such an acceleration of the blood-brain transport would not be dependent on the glutamine synthesis. If inhibition of the glutamine synthesis were to prevent such a hypothetical increase in the blood-brain transport, this would support the gamma-glutamyl cycle hypothesis.

The present work suggests a mechanism of blood-brain transport of LNAA that involves an influx of LNAA into brain in exchange for an efflux of glutamine and other LNAA. Consequently, it would be of interest to measure the cerebral arterio-venous differences in the amino acid concentrations in rats after ammonia infusion and after PCA, and in humans with chronic liver failure and HE. Such an investigation has been performed in patients with chronic liver cirrhosis without HE. This study did not show an efflux of glutamine out of brain. Despite high ammonia levels in patients with liver cirrhosis and slight or no HE, we did not find increased LNAA concentrations in CSF after an ammonia load. This may be due to species differences. In order to answer this question we plan to perform ammonia tolerance tests in patients with liver cirrhosis and pronounced HE and study the changes in the LNAA concentrations in CSF.

In humans and in experimental animals with liver failure, disturbances in the indoleamine and catecholamine concentrations in the central nervous system have been reported. These changes have been suggested to contribute to HE. It is not demonstrated, however, that the observed changes really play a role in the development and the symptomatology of HE. Studies on the relationship between neurochemical and behavioural changes in experimental animals may shed light on this. Besides, the number of known neurotransmitters is increasing and we do not know their importance for the development of HE.

Many clinical trials have been performed to study the effect of BCAA treatment on HE. Some investigators have demonstrated a good effect of BCAA on acute and chronic HE, whereas others have not been able to confirm their findings. This highlights the difficulties

in performing clinical trials. The etiology of liver disease should be well defined. The grade of HE of the patients when entering the trials, the precipitating factors, the exclusion criteria, the length of the treatment, the number of patients in the trials are some factors that must be considered when the results are evaluated. In most clinical trials of BCAA therapy in liver failure, BCAA infusion has been compared to conventional therapy. This is in my opinion wrong: BCAA treatment should be compared to conventional amino acid infusion. The present investigation supports a special and separate role of ammonia, and therapy directed at lowering the hyperammonemia should be either equal in both treatment groups or omitted. Such a trial comparing BCAA and conventional amino acids in HE is presently ongoing in our department.

Clinical investigations have also suggested that BCAA could have a positive effect on the protein metabolism. How this effect is mediated is not known. Up to now, enough attention has not been focused on the effect of BCAA on the protein metabolism in treatment of liver failure.

Another question is whether BCAA can stimulate liver function and liver regeneration. The improvement of liver function and liver regeneration may determine the final outcome of a patient in liver failure. The influence of different nutritional regimens on liver function and liver regeneration must be further evaluated.

The importance of hyperammonemia for the LNAA and neurotransmitter changes in CNS in HE, shown in this work, suggests that lowering of plasma ammonia besides infusion of BCAA may affect neurotransmission.

Plasmapheresis has been tried in fulminant hepatic failure (110). It removes ammonia and other toxic substances from the plasma. The experience of this treatment is limited, but it is a treatment that should be tried more frequently.

In children with inborn errors of urea synthesis, long-term treatment with arginine, sodium benzoate and sodium phenylacetate has managed to reduce the plasma ammonia concentrations and maintain normal growth (111). Whether this treatment will lower ammonia and be of value for patients with chronic liver disease should be evaluated.

Further trials with a nutritional therapeutic approach to patients in liver failure and with HE are thus needed.

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FACTORS INFLUENCING THE CONCENTRATIONS OF THE
LARGE NEUTRAL AMINO ACIDS IN THE BRAIN AND IN
THE CSF OF DOGS AFTER PORTACAVAL ANASTOMOSIS¹

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ABSTRACT

Portal-systemic shunting of blood is associated with hyperammonemia, an increased glutamine concentration in brain, an altered plasma neutral amino acid pattern, and high levels of several of the large neutral amino acids in brain. Since some of these amino acids are precursors for neurotransmitters and for other potentially neuroactive substances, high CNS levels of these amino acids may contribute to development of encephalopathy. In order to determine the relative importance of changes in brain glutamine levels and changes in competition among the neutral amino acids for blood-brain transport, we measured the concentrations of the large neutral amino acids in plasma, cisternal cerebrospinal fluid and in brain tissue from various regions of dogs after end-to-side portacaval shunt. Although the changes in CSF amino acid levels were somewhat correlated with altered amino acid plasma competitor ratios, better correlations were observed with the elevation of CSF glutamine. These results suggest a model of blood-brain amino acid transport in which a high level of glutamine in brain extracellular fluid competes with other neutral amino acids for efflux from brain, thus raising brain amino acid levels after portal-systemic shunting.

INTRODUCTION

One of the interesting problems in the study of hyperammonemia, liver failure and portal-systemic encephalopathy is the relationship of changes in brain of the neurotransmitter-precursor amino acids to the development of encephalopathy. The amino acids of particular interest are tyrosine, phenylalanine and tryptophan, since they are involved in the synthesis in brain of catecholamines, serotonin and of potential false transmitters such as octopamine or tryptamine [7,5]. These amino acids, together with leucine, isoleucine, valine, methionine, threonine and histidine, comprise the group of large neutral amino acids (LNAA) which share a common blood-brain transport system. In both humans and experimental animals with hepatic failure, many LNAA accumulate in the CNS to a greater extent than the changes in plasma concentrations of those amino acids would seem to predict [18,11,15]. The reason for this "excess" accumulation of LNAA in the CNS of patients or experimental animals with portal-systemic shunting and hyperammonemia has not been completely explained, although data have been presented which point to a role for elevated glutamine concentrations in the CNS in altering LNAA transport at the blood-brain barrier [1,12,13]. In the following study in dogs, we compared the changes after portacaval anastomosis (PCA) in LNAA concentrations in plasma, cisternal CSF and in brain tissue. The latter was obtained only at sacrifice, but the plasma and CSF were sampled sequentially after PCA in order to follow changes over time. The results point to a significant role of elevated brain GLN as a competitor for efflux of the LNAA from the brain to the blood.

MATERIAL AND METHODS

Sixteen conditioned dogs weighing 9.5-23.9 kg were studied. Twelve dogs were anesthetized with sodium pentobarbital and subjected to an end-to-side portacaval shunt. The anastomosis was done with 5-0 Prolene sutures using the portal vein stump above the last splanchnic branch. After the operation 5% dextrose and saline solution were infused for 24 hours and pencillin and streptomycin were given. All dogs were allowed chow ad libitum. Weight, but not food intake, was monitored. The remaining four unoperated dogs served as controls.

From previous experience in this laboratory [24] with this experimental model, we anticipated that the development of encephalopathy in each dog would be difficult to predict. We intended to sacrifice similar numbers of dogs in Stage I (mild), Stage II (moderate) and Stage III (severe) encephalopathy. Therefore, starting from the second postoperative day two observers independently rated the dogs for encephalopathy. The parameters used for this evaluation were spontaneous activity, ataxia, salivation, ease of arousal, response to foot pinching and abnormalities of gait. The animals were clinically graded as follows:

- Grade 0: Indistinguishable from normal (no animals were sacrificed in this condition).
- Grade I: Less lively, with delayed responses.
- Grade II: Hypersalivation; hyperactive movements; walking close to the walls with an unsteady gait.
- Grade III: Excessive salivation; sleeping most of the time; when forced

to walk, walking "into" the walls; standing with great difficulty; very slow response to foot-pinch; coma.

Samples were obtained as follows: Venous blood and CSF from the cisterna magna were obtained from dogs, anesthetized with pentobarbital, preoperatively and at 3-week intervals thereafter. Heparinized blood was immediately cooled on ice and centrifuged, and plasma was either processed immediately or kept frozen at -70°C . The plasma amino acid concentrations were determined by using a Beckman 121 MB amino acid analyzer with automatic integration. The plasma was deproteinized by mixing 0.5 ml plasma with 1.5 ml of 5% sulfosalicylic acid (pH 1.8, adjusted with LiOH) containing as an internal standard 133 nmol/ml thienylalanine. CSF samples were prepared by mixing 0.9 ml CSF with 0.1 ml 37.5% sulfosalicylic acid, pH 2.0, containing 500 nmol/ml thienylalanine. After that all samples were centrifuged (20,000 x g, 20 min, 4°C) and passed through a 0.4 μm filter (Millipore Corp.) and frozen at -70°C until analysis.

At the time of sacrifice, the dogs were anesthetized with pentobarbital and blood and CSF samples were taken. The vault of the skull was removed by an electric saw and the brain disconnected at the junction with the spinal cord and rapidly transferred to ice-cold normal saline. The brain was then put on its dorsal surface on an ice-cold, saline-soaked paper towel. Nine regions of the brain were isolated separately and in the following order: hypothalamus, diencephalon, cerebellum, medulla, pons, mesencephalon, cortex, caudate nucleus, and hippocampus. The brain samples were immediately frozen in liquid nitrogen and stored at -70°C until the

analysis. On the average, the samples were frozen within 5 min after the brain was removed. Hypothalamus was dissected by making a cut at a depth of approximately 5 mm from just anterior to the optic chiasma to the posterior aspect of the mamillary body bounded laterally by the choroid fissure. A sample was next taken from the diencephalon just dorsal to the hypothalamus. Cerebellum was then removed and a portion (1-2 g) taken for analysis. Pons was separated from the medulla at the pontine fissure. Mesencephalon was removed from the rest of the brain stem by a vertical cut just anterior to the superior colliculus. Next, the brain was divided into the two hemispheres and both caudate nuclei were removed from the lateral walls of the lateral ventricles. Hippocampal tissue was removed from the temporal recessus of the lateral walls of the lateral ventricles and cortical samples were taken from the temporoparietal areas.

The brain samples were homogenized in three volumes of 5% sulfosalicylic acid, pH 1.4, containing 133 nmol/ml of thienylalanine as internal standard. The samples were then handled in the same way as for plasma and CSF with the exception that each sample was analyzed twice, i.e., once at the 1:4 dilution of the homogenate for the low concentration amino acids, and then after a further 1:25 dilution to measure the high concentration amino acids glutamate and glutamine. Plasma, brain and CSF were analyzed for threonine (THR), glutamine (GLN), valine (VAL), methionine (MET), isoleucine (ILE), leucine (LEU), tyrosine (TYR), phenylalanine (PHE), tryptophan (TRP), histidine (HIS), and (in brain only) alanine (ALA) and glutamate (GLU).

One aim of this study is to determine whether the rise in the concentrations of various AAs in the CNS could be accounted for solely by

changes in circulating LNAA levels, the manner in which the circulating amino acid levels should be expressed is of importance. Studies in rats have demonstrated that the LNAA (THR, VAL, MET, ILE, LEU, TYR, PHE, HIS, TRP) compete for a common blood-brain transport system [17,25]. Glutamine can apparently be transported by this system also, but has a very low affinity for it [17,19]. Each LNAA has a characteristic affinity for the transport system, with some amino acids (e.g., LEU, PHE) competing more effectively for transport than others (e.g., VAL, THR). Studies from this laboratory in dogs have clearly demonstrated the effects of competition among plasma amino acids on CSF levels of LNAA [24]. Studies in dogs by others have suggested that the rank-order of transport K_m 's (affinities) is similar to that in rats [25]; however, no blood-brain transport K_m values are available for normal dogs or in dogs after PCS.

We expressed the availability of a particular amino acid for blood-brain transport in a form of the Michaelis-Menten kinetic formula which accounted for the competition among plasma amino acids due both to their different concentrations in plasma and also to their presumed relative affinities (K_m). The plasma competitor ratios (PCR) of the studied amino acids were calculated in a way similar to the brain influx rate used by Fernstrom and Faller [6] with the modification that the V_{max} of transport was considered to be 100% as previously described [8]. One interpretation of uptake expressed in this fashion is that the calculated values represent the fraction of maximal transport activity expended on a particular amino acid. It is assumed, in making these calculations, that blood-brain LNAA transport does not greatly differ in dogs and rats and that PCR does not alter the

affinities (K_m) of the amino acids for the transport system. The PCR were calculated as follows:

$$PCR_x = \frac{AA_x}{K_{m_x} [1 + AA/K_m] + AA_x} \times 100\%$$

where AA_x is the plasma concentration of the NAA_x; K_{m_x} is the transport K_m of AA_x ; K_m is the transport K_m of competing NAA; AA is the plasma concentrations of competing NAA; and V_{max} is the transport $V_{max} = 100\%$. The K_m 's of transport for each neutral amino acid from Pardridge and Mietus [19] and Pardridge and Oldendorf [20] were as follows: THR = 730 nmol/ml; VAL = 510 nmol/ml; ILE = 250 nmol/ml; LEU = 100 nmol/ml; MET = 180 nmol/ml; TYR = 150 nmol/ml; PHE = 110 nmol/ml; and HIS = 240 nmol/ml. Tryptophan concentrations were not included in the calculation since the actual amount of "free" or non-albumin-bound tryptophan was not determined. This fraction of the plasma tryptophan is relatively small and therefore exerts only slight competitive effect.

Statistical analysis of differences among serial samples of plasma and CSF was by analysis of variance and Newman-Keuls test. Correlation was tested by Pearson product-moment correlation. Comparison of amino acid concentrations in brain regions of control dogs and dogs killed in various stages of encephalopathy is by the non-parametric Kruskal-Wallis test.

RESULTS

The interval from PCA until development of encephalopathy and sacrifice was highly variable, ranging from 21 to 213 days. The mean overall weight

loss at time of sacrifice was $26 \pm 3\%$. The percentage weight loss was proportional to the grade of encephalopathy at time of sacrifice: $20 \pm 4\%$ in grade I, $29 \pm 4\%$ in grade II, and $34 \pm 4\%$ in grade III. At time of sacrifice six dogs were rated in grade I, 3 dogs in grade II, and 3 dogs in grade III. It should be noted that, due to the unpredictability of the course of encephalopathy after PCA in dogs, it proved difficult to achieve the goal of equal numbers of dogs in each stage of encephalopathy. In practice, those dogs which deteriorated quickly to stage II and III were sacrificed first, whereas those dogs which showed little tendency to develop severe encephalopathy were sacrificed at later times (up to several months) after surgery and were mostly in stage I.

Table 1 compares the changes in plasma LNAA concentrations or competitor ratios at intervals after PCA with observed LNAA concentrations in cisternal CSF. The changes in the plasma amino acid pattern after PCA were similar to those previously reported [24] and characterized by increased concentrations of histidine and of the aromatic amino acids tyrosine and phenylalanine. The calculated plasma competitor ratios (Table 1) also changed after PCA in directions similar to the absolute plasma amino acid concentrations. Note that preoperatively the percentage of calculated transport activity was greatest for LEU and PHE, reflecting their high affinities for transport (low K_m). After PCA the calculated ratios for PHE and TYR approximately doubled, representing the greatest increase of any of the LNAA in this parameter. The calculated competitor ratios for the BCAA decreased to a greater extent than their absolute concentrations in plasma.

The CSF concentration of GLN, presumably the product of cerebral ammonia

detoxification with glutamic acid, rose after PCA to a much greater degree than in plasma. The increases in the concentration of PHE, TYR, HIS and TRP in CSF greatly exceeded the change either in absolute plasma levels or in the calculated competitor ratios.

In all brain regions of dogs with PCA, the concentrations of GLN, TYR, PHE, TRP and HIS were several-fold higher than control, while MET and LEU were increased to a lesser extent. The regional concentrations of alanine (ALA) and of glutamic acid (GLU), the precursor for GLN synthesis, are also shown in Table 2 for comparison, although they are not substrates for the LNAA transport system of the blood-brain barrier. There was no consistent statistical difference in regional amino acid levels between dogs sacrificed in encephalopathy grade I as compared with dogs in grades II and III combined. Combining the data of dogs in grade II and III was necessary to achieve sufficient numbers for statistical analysis.

We tested whether the concentration of each of the LNAA in CSF was more closely correlated to the plasma competitor ratio of that amino acid or to the CSF concentration of GLN (Table 3). For these correlations, 45 data-pairs were considered. Significant correlations were observed between the CSF concentrations of THR, TYR, PHE and HIS and their respective plasma competitor ratios. Significant correlations were also found between the CSF concentration of GLN and that of MET, LEU, TYR, PHE and HIS. For all CSF amino acids except THR, the correlation with CSF GLN was better (higher value of the correlation coefficient (r)) than with the plasma competitor ratio, suggesting that CSF LNAA concentrations are more strongly influenced by the CNS concentration of GLN than by the availability of LNAA for

transport from the circulation. The relationship between CSF GLN and the four LNAA with the best correlations are shown graphically in Figure 1.

A similar comparison of correlations was performed between the regional brain concentration of each LNAA and either the plasma competitor ratio or the concentration of GLN in that region (Table 4). For these correlations, all 16 dogs were considered. As in the CSF, the best correlations were observed for MET, PHE, TYR and HIS. For MET and HIS, the better correlations were clearly with the brain GLN concentration; whereas, for PHE and TYR, their brain concentrations correlated about equally well both with the plasma competitor ratio and the brain GLN level.

The correlation between CSF GLN and regional GLN was tested in the twelve dogs with a PCS. The controls were omitted in this case since including them would have markedly improved all the correlations. A statistically significant correlation was obtained only for cortex ($r = 0.645$, $p < 0.05$; Fig. 2) and for cerebellum ($r = 0.593$, $p < 0.05$). This result may reflect the fact that cerebral cortex and cerebellum constitute two of the largest brain regions in dogs and, hence, diffusion of amino acids from those regions may have considerable influence on the composition of CSF.

DISCUSSION

The lack of correlation in the present studies between encephalopathy grade and brain levels of amino acids which are known or thought to be involved in synthesis of true or false neurotransmitters would seem to weaken the hypothesis that changes in brain amino acid levels are involved in encephalopathy. However, it should be noted that the design of the experiment may have resulted in significant bias if there was some difference in "sensitivity" to high brain LNAA levels in those dogs which exhibited encephalopathy at early or late times after the PCA. We have no evidence, however, that such differences in sensitivity exist.

Models of blood-brain amino acid transport consider the existence of at least three compartments: (a) blood or plasma, (b) brain extracellular fluid and the CSF, and (c) the intracellular fluid of brain cells [22]. Amino acids in a cerebral capillary must be transported across the two plasma membranes of the capillary endothelial cell in order to enter the extracellular fluid where they are then available to the transport systems of the brain cells themselves. Similarly, to exit from brain, amino acids must cross the brain cell membrane into the extracellular fluid and then must cross the two capillary membranes in order to reach the circulation. The total capillary surface area is very small compared to the sum of brain cell membranes. It has been proposed that the limiting factor in blood-brain transport of amino acids (as well as of other substances requiring carrier-mediated transport) is transport across the capillary membranes [21].

In the present studies, with the exception of THR, the magnitude of the increase or decrease in absolute plasma LNAA levels was not the same as the changes in concentration of amino acids in CSF or in brain. The magnitude of change in the calculated plasma competitor ratios for each LNAA was also considerably less than the change in CSF or brain LNAA after PCA. These observations strongly suggest that some factor other than the change in plasma LNAA concentrations was responsible for the rise in CSF and brain LNAA levels after PCA.

Elevated concentrations of GLN in lumbar CSF are commonly found in patients with encephalopathy resulting from liver disease [9]. The synthesis in brain of GLN from ammonia and glutamic acid apparently occurs very rapidly [4] and primarily in glial astrocytes, where the enzyme glutamine synthetase is located [16]. The elevated CSF GLN concentration in patients almost certainly reflects accelerated detoxification of ammonia in brain secondary to hyperammonemia [14]. In the present studies, the CSF GLN concentration rose from levels which were less than those in plasma preoperatively to levels which were markedly greater than in plasma (Table 1). This change in the CSF/plasma GLN ratio is strong evidence that transport of GLN from extracellular fluid to blood across the capillary barrier was slow compared to the rate of GLN synthesis, at least until CSF GLN reached a much higher level. Presumably, at the higher level, synthesis of GLN was matched by the rate of removal of GLN from the ECF. The present studies suggest that competition for transport from ECF to blood among GLN and the other LNAA can be an important determinant of brain LNAA levels in liver disease.

The increased CSF concentration of several of the LNAA was better correlated with the increased CSF GLN concentration than with the change in the plasma competitor ratios of those amino acids (Table 3). This does not imply that competition among plasma neutral amino acids for transport across the blood-brain barrier is not an important determinant of brain amino acid levels. On the contrary, Smith, et al. [24] showed that infusing a solution rich in the BCAA could drastically reduce the CSF levels of the other LNAA in dogs with portal-systemic encephalopathy. The lower degree of correlation between plasma competitor ratios and CSF LNAA levels suggests that, under the pathological conditions of the present study, the GLN concentration in the CNS was a more important determinant of the concentrations of the LNAA in the brain or CSF.

The most direct means by which a high concentration of GLN in the brain ECF might affect the levels of other LNAA in brain ECF is for GLN to compete with the other LNAA for transport out of brain. If transport of the LNAA across the capillary endothelial barrier in the blood-to-brain direction is, as is thought, primarily determined by competition, then it is very likely that transport in the brain-to-blood direction is primarily determined by competition also. Increased concentrations of GLN in CSF would inhibit the efflux of other LNAA until sufficient concentrations of the other LNAA had accumulated in the ECF to overcome this competition for transport out of brain. At this new steady state the relationship between influx and efflux of the LNAA would be restored to more normal levels. According to this hypothesis, the rise in LNAA concentrations in the ECF would be a necessary and passive consequence of the increase in GLN concentration in the same

compartment.

The rise in brain intracellular concentrations of the LNAA may be explained by assuming that the amino acid transport systems of brain cells include concentrative mechanisms which actively "pump" amino acids into brain cells against their concentration gradients [23]. If it is further assumed that these transport systems are capable of establishing a gradient with a fixed inside/outside concentration ratio of 3:1 to 4:1, then the rise in brain LNAA concentrations would follow as a simple consequence of the rise in ECF LNAA concentrations. In the present studies, the observation that the changes in CSF and in brain tissue amino acids were of similar magnitude after PCA is consistent with this assumption.

It should be recalled that the metabolic fate of the various LNAA will affect their final concentrations intracellularly. None of the LNAA, except GLN and perhaps a small amount of TYR (from PHE), can be synthesized in brain. The brain can metabolize the leucine and probably the other BCAA [3]. It is therefore likely that the small change in brain levels of the BCAA after PCA, as compared with the greater increase in the AAA, is due in part to more rapid metabolism of the BCAA. It is nonetheless notable that brain and CSF LEU concentrations rose after PCA even though plasma LEU fell.

This hypothetical sequence of events does not require that the rate of transport of LNAA across the blood-brain barrier actually be increased in the presence of hyperammonemia and portal-systemic shunting. Changes in the rate of such transport have been observed in rats after PCA [11,20,21], but attempts to demonstrate accelerated blood-brain transport *in vivo* after PCA have not been successful [10]. If the unidirectional flux of LNAA from

blood to brain was, in fact, increased in dogs with PCA, then the principal effect of this would be to accelerate the accomplishment of the new steady state levels of LNAA in the ECF, which in turn is determined by the ECF GLN concentration.

These various factors affecting LNAA levels after PCA in CSF or in brain tissue are summarized schematically in Figure 3. Compared to normal (A), the only change shown immediately after PCA (B) is a rise in blood ammonia. In actuality the changes in blood ammonia and in plasma LNAA concentrations probably proceed simultaneously, but for the purpose of discussion we will consider these two processes as distinct. As a consequence of hyperammonemia, the synthesis of glutamine in astrocytes is stimulated, resulting in increased brain GLN concentrations. Panel (B) also represents a hypothetical but possibly observable moment when brain GLN is elevated, yet the CSF GLN level has not yet begun to rise. According to the role of GLN in blood-brain LNAA transport proposed previously in this discussion, no change in CSF or brain LNAA levels should occur before CSF GLN becomes elevated.

In panel (C) the characteristic changes in plasma LNAA after PCA alter the plasma competitor ratios and thus the ratio of AAA/BCAA which can be transported into brain. The elevated ECF GLN now competes for brain-to-blood transport with the other LNAA, resulting in higher ECF and thus brain levels of the AAA. Brain levels of BCAA are little affected both because the influx of BCAA is somewhat reduced and because these amino acids are metabolized in brain.

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FOOTNOTE

- 1 In conducting this research, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" as promulgated by the Committee of Care and Use of Laboratory Animals in the Institute of Laboratory Animal Resources, National Research Council.

TABLE 1: Changes in plasma amino acid concentrations, calculated plasma competitor ratios, and cisternal CSF concentrations at various times after PCA.

Amino Acid GLN	Plasma (nmol/ml $\pm$ SEM)				Plasma Competitor Ratio (% $V_{max} \pm$ SEM)				CSF (nmol/ml $\pm$ SEM)			
	pre-op 671 $\pm$ 26	3 wk 1005 $\pm$ 60	6 wk 1068 $\pm$ 80	9 wk 961 $\pm$ 112	pre-op NC	3 wk	6 wk	9 wk	pre-op 447 $\pm$ 13	3 wk	6 wk	9 wk
THR	203 $\pm$ 29	167 $\pm$ 16 (-18%)	187 $\pm$ 21 (-8%)	162 $\pm$ 25 (-20%)	6.3 $\pm$ 0.7	5.0 $\pm$ 0.4 (-21%)	5.4 $\pm$ 0.6 (-14%)	5.0 $\pm$ 0.9 (-21%)	49 $\pm$ 5	42 $\pm$ 5 (-14%)	47 $\pm$ 5 (-4%)	44 $\pm$ 5 (-10%)
VAL	143 $\pm$ 13	114 $\pm$ 14 (-20%)	113 $\pm$ 16 (-21%)	117 $\pm$ 16 (-18%)	7.5 $\pm$ 0.2	4.8 $\pm$ 0.3 (-36%)	4.6 $\pm$ 0.3 (-39%)	4.9 $\pm$ 0.5 (-35%)	11 $\pm$ 1	12 $\pm$ 1 (+9%)	14 $\pm$ 1 (+27%) ^a	16 $\pm$ 2 (+45%) ^a
ILE	37 $\pm$ 6	37 $\pm$ 5 (0)	34 $\pm$ 5 (-8%)	37 $\pm$ 2 (0)	3.4 $\pm$ 0.2	3.0 $\pm$ 0.3 (-12%)	2.3 $\pm$ 0.2 (-33%)	2.7 $\pm$ 0.4 (-21%)	4 $\pm$ 1	4 $\pm$ 1 (0)	5 $\pm$ 1 (+25%)	5 $\pm$ 1 (+25%)
LEU	91 $\pm$ 15	82 $\pm$ 10 (-10%)	78 $\pm$ 11 (-14%)	79 $\pm$ 10 (-13%)	22.7 $\pm$ 0.9	17.9 $\pm$ 1.2 (-21%)	15.7 $\pm$ 0.9 (-31%)	17.1 $\pm$ 1.7 (-25%)	13 $\pm$ 1	14 $\pm$ 1 (+8%)	17 $\pm$ 1 (+31%)	19 $\pm$ 3 (+46%) ^a
MET	45 $\pm$ 3	48 $\pm$ 4 (+7%)	59 $\pm$ 9 (+31%)	42 $\pm$ 6 (-7%)	6.9 $\pm$ 0.4	5.8 $\pm$ 0.3 (-16%)	7.1 $\pm$ 0.7 (+3%)	5.8 $\pm$ 0.5 (-16%)	8 $\pm$ 1	18 $\pm$ 3 (+125%) ^a	23 $\pm$ 3 (+187%) ^a	21 $\pm$ 3 (+162%) ^a
TYR	32 $\pm$ 4	75 $\pm$ 7 (+134%) ^a	98 $\pm$ 10 (+206%) ^a	82 $\pm$ 9 (+156%) ^a	5.7 $\pm$ 0.4	10.8 $\pm$ 0.4 (+89%)	12.1 $\pm$ 0.3 (+112%)	11.3 $\pm$ 0.8 (+98%)	9 $\pm$ 1	32 $\pm$ 3 (+256%) ^a	53 $\pm$ 6 (+489%) ^a	42 $\pm$ 7 (+367%) ^a
PHE	43 $\pm$ 4	102 $\pm$ 10 (+137%) ^a	132 $\pm$ 20 (+207%) ^a	109 $\pm$ 10 (+153%) ^a	10.3 $\pm$ 0.5	20.1 $\pm$ 0.7 (+95%)	21.2 $\pm$ 1.5 (106%)	20.6 $\pm$ 1.9 (+100%)	12 $\pm$ 1	43 $\pm$ 3 (+258%) ^a	72 $\pm$ 8 (+500%) ^a	61 $\pm$ 14 (+408%) ^a
HIS	64 $\pm$ 3	93 $\pm$ 7 (+45%) ^a	117 $\pm$ 11 (+83%) ^a	99 $\pm$ 6 (+55%) ^a	7.4 $\pm$ 0.4	8.6 $\pm$ 0.4 (+16%)	9.7 $\pm$ 0.4 (+31%)	9.0 $\pm$ 0.9 (+22%)	13 $\pm$ 1	34 $\pm$ 3 (+162%) ^a	49 $\pm$ 3 (+277%) ^a	44 $\pm$ 10 (+238%) ^a
TRP	59 $\pm$ 4	66 $\pm$ 9 (+12%)	83 $\pm$ 10 (41%)	60 $\pm$ 9 (+2%)	NC	NC	NC	NC	5 $\pm$ 1	14 $\pm$ 1 (+180%) ^a	18 $\pm$ 2 (+260%) ^a	17 $\pm$ 3 (+240%) ^a

Percent change from pre-op indicated beneath values.

NC = not calculated (see text).

Number of dogs = pre-op, 12; 3 wk, 12; 6 wk, 9; 9 wk, 5.

^a Significantly different from controls; $p < 0.01$

TABLE 2: Regional concentrations of amino acids in the brain of dogs before and after portacaval anastomosis.

Brain Region	Grade of Encephalopathy	Amino Acid											
		THR	GLU	GLN	ALA	VAL	MET	ILE	LEU	TYR	PHE	TRP	HIS
Diencephalon	Controls (4)	262	9039	4670	371	98	42	25	57	42	35	18	160
	I (6)	154 ^a	8125	22320 ^a	381	95	92 ^a	29	88 ^a	235 ^a	244 ^a	65 ^a	544 ^a
	II + III (6)	157 ^a	8654	18184 ^a	378	122	62 ^a	35 ^a	123 ^a	221 ^a	308 ^a	77 ^a	410 ^a
Hypothalamus	Controls	214	4359	3498	262	72	29	15	51	37	35	23	61
	I	144	4861	13577 ^a	279	70	65 ^a	15	49	158 ^a	166 ^a	49 ^a	206 ^a
	II + III	132 ^a	5089	16463 ^a	425	87	62 ^a	21	77 ^a	144 ^a	196 ^a	52 ^a	194 ^a
Medulla	Controls	263	5809	3468	421	107	44	35	63	51	50	22	133
	I	165 ^a	6048	17941 ^a	428	111	63 ^a	38	84	247 ^a	291 ^a	63 ^a	420 ^a
	II + III	124 ^a	5667	18847 ^a	365	136	86 ^a	33	103	210 ^a	321 ^a	68 ^a	368 ^a
Pons	Controls	278	5952	2984	406	83	37	22	60	48	50	22	115
	I	192	4786	11734 ^a	375	86	105 ^a	19	73	246 ^a	322 ^a	63 ^a	376 ^a
	II + III	141 ^a	5191	19149 ^{ab}	458	90	86 ^a	27	100 ^a	319 ^a	321 ^a	68 ^a	199 ^a
Cortex	Controls	281	10654	6174	590	126	61	33	86	55	52	20	108
	I	145 ^a	9460	23506 ^a	605	231	89 ^a	34	100	260 ^a	295 ^a	76 ^a	410 ^a
	II + III	128 ^a	9541	32773 ^a	596	238	69 ^a	36	134 ^a	247 ^a	266 ^a	76 ^a	303 ^a
Caudate Nucleus	Controls	356	11510	7031	481	93	45	24	72	48	47	24	103
	I	184 ^a	10729	23709 ^a	548	73	101 ^a	27	89	234 ^a	230 ^a	67 ^a	349 ^a
	II + III	141 ^a	10937	21287 ^a	906	115	71 ^a	42	121	206 ^a	280 ^a	73 ^a	294 ^a
Hippocampus	Controls	362	8451	4492	733	98	52	24	71	51	41	20	172
	I	189 ^a	8273	16954 ^a	753	88	83 ^a	23	84	234 ^a	231 ^a	67 ^a	544 ^a
	II + III	122 ^a	9115	16894 ^a	755	116	65 ^a	36	111	203 ^a	273 ^a	63 ^a	509 ^a
Mesencephalon	Controls	291	6239	3478	498	153	41	22	63	48	45	21	140
	I	178 ^a	6217	18516 ^a	420	115	99 ^a	32	85	248 ^a	268 ^a	63 ^a	429 ^a
	II + III	128 ^a	6637	19125 ^a	474	130	72 ^a	35	107 ^a	223 ^a	296 ^a	65 ^a	444 ^a
Cerebellum	Controls	345	8561	6566	370	112	51	27	95	59	61	27	120
	I	187 ^a	8753	17748 ^a	415	113	112 ^a	20	90	237 ^a	293 ^a	67 ^a	288 ^a
	II + III	154 ^a	8706	23030 ^a	513	134	79	22	131	232 ^a	351 ^a	61 ^a	292 ^a

Results are given as the median since a non-parametric test was used for the calculations. a significantly different from the controls; $p < 0.05$ at least.

b Significant difference between encephalopathy grade I vs. grade II + III; $p < 0.05$ at least.

TABLE 3. Pearson product-moment correlations between CSF concentrations of LNAAs and either the plasma competitor ratio (PCR) or CSF GLN.

Amino Acid	versus PCR	versus CSF GLN
	(r)	(r)
THR	0.433 ^a	0.033
VAL	0.170	0.340
MET	-0.138	0.654 ^b
ILE	0.057	0.144
LEU	0.035	0.527 ^b
TYR	0.720 ^b	0.950 ^b
PHE	0.790 ^b	0.914 ^b
HIS	0.439 ^a	0.904 ^b

^a $p < 0.01$

^b $p < 0.001$

TABLE 4: Correlation of regional brain concentrations of several LWA with the plasma competitor ratio and with the regional brain GLN concentration.

METHIONINE			
Region	PCR vs Brain Concentration (r)	Brain GLN vs Brain Concentration (r)	
Dien	0.042	0.590 ^a	
Hypo	0.254	0.482	
Med	-0.168	0.618 ^b	
Pons	-0.147	0.369	
Cortex	0.185	0.536 ^a	
CN	0.173	0.513 ^a	
Hippo	-0.135	0.461	
Mesen	0.002	0.601 ^a	
Cereb	0.208	0.676 ^b	

PHENYLALANINE			
Region	PCR vs Brain Concentration (r)	Brain GLN vs Brain Concentration (r)	
Dien	0.951 ^c	0.948 ^c	
Hypo	0.889 ^c	0.952 ^c	
Med	0.943 ^c	0.980 ^c	
Pons	0.903 ^c	0.673 ^b	
Cortex	0.954 ^c	0.842 ^c	
CN	0.972 ^c	0.957 ^c	
Hippo	0.961 ^c	0.964 ^c	
Mesen	0.945 ^c	0.946 ^c	
Cereb	0.924 ^c	0.960 ^c	

TYROSINE			
Region	PCR vs Brain Concentration (r)	Brain GLN vs Brain Concentration (r)	
Dien	0.901 ^c	0.950 ^c	
Hypo	0.803 ^c	0.924 ^c	
Med	0.852 ^c	0.927 ^c	
Pons	0.767 ^c	0.694 ^c	
Cortex	0.929 ^c	0.855 ^c	
CN	0.904 ^c	0.902 ^c	
Hippo	0.899 ^c	0.929 ^c	
Mesen	0.886 ^c	0.914 ^c	
Cereb	0.857 ^c	0.928 ^c	

HISTIDINE			
Region	PCR vs Brain Concentration (r)	Brain GLN vs Brain Concentration (r)	
Dien	0.670 ^b	0.861 ^c	
Hypo	0.467	0.891 ^c	
Med	0.567 ^a	0.938 ^c	
Pons	0.703 ^b	0.395	
Cortex	0.590 ^a	0.680 ^b	
CN	0.534 ^a	0.924 ^c	
Hippo	0.563 ^a	0.941 ^c	
Mesen	0.540 ^a	0.933 ^c	
Cereb	0.540 ^a	0.953 ^c	

^a $p < 0.05$

^b $p < 0.01$

^c $p < 0.001$

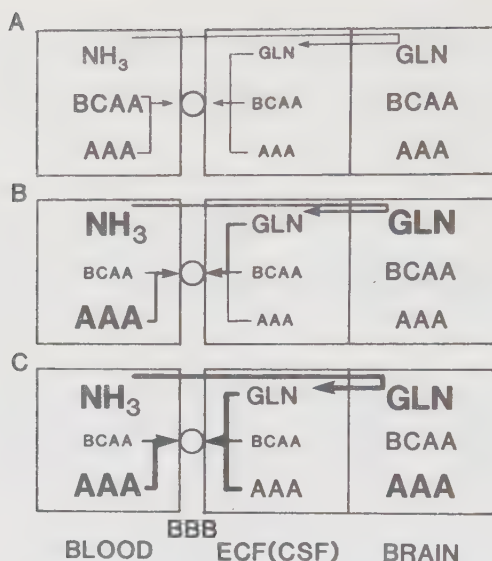


Fig. 3

Proposed scheme reflecting events which occur after PCA in the blood, CSF or extracellular fluid (ECF) and brain compartments with respect to ammonia (NH₃), BCAA and AAA. Panel A shows normal pre-operative levels of NH₃ and amino acids in blood plasma, CSF and brain tissue. Smaller type-face in ECF reflects much lower concentrations in that compartment. Panel B represents a hypothetical period after creation of the PCA when blood ammonia is elevated, thus raising the brain GLN concentration, but before the CSF concentration of GLN has risen and thus before blood-brain NAA transport has been affected. In Panel C, plasma levels of AAA are elevated while those of BCAA are decreased, thus altering plasma competitor ratios. High GLN in the ECF plus altered competition for transport result in greatly increased brain AAA concentrations.

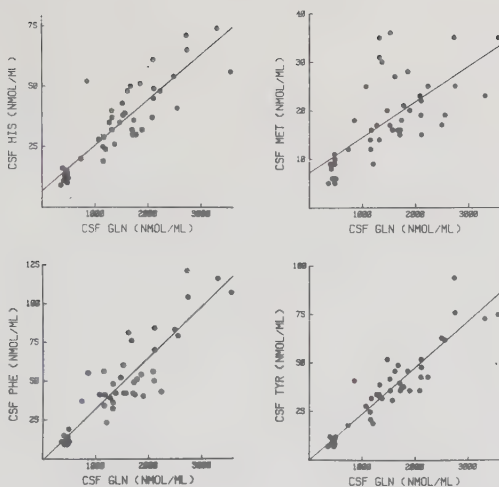


Fig. 1

Relationship between the CSF concentration of GLN and the CSF concentrations of HIS, MET, PHE and TYR. The data points shown represent pre-operative samples, clustered at lower left of each panel, and all subsequent CSF samples obtained on all dogs. Correlation coefficients are shown in Table 3.

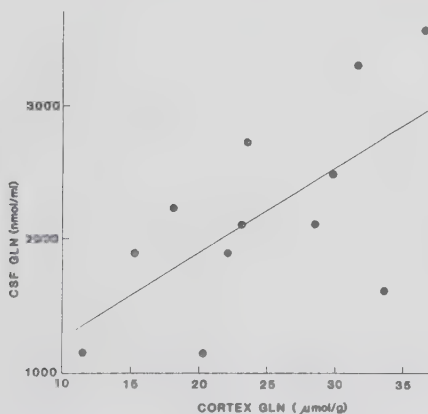


Fig. 2

The relationship of cisternal CSF GLN to GLN in cortex in the twelve dogs with PCA at time of sacrifice.

Methionine Sulfoximine Prevents the Accumulation of Large Neutral Amino Acids in Brain of Portacaval-Shunted Rats

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Abstract: Portal-systemic shunting and hyperammonemia lead to an accumulation of the large neutral amino acids in brain and apparently alter transport of neutral amino acids across the blood-brain barrier. It has been proposed that portal-systemic shunting leads to a high brain concentration of glutamine, a product of cerebral ammonia detoxification, and thereby affects the transport of other neutral amino acids across the blood-brain barrier. To test this hypothesis, rats with a portacaval shunt were treated with L-methionine-*dl*-sulfoximine (MSO), an inhibitor of glutamine synthesis. Treatment with MSO resulted in lower concentrations of the neutral amino acids

in brain of portacaval-shunted rats and a higher brain ammonia concentration, compared with untreated shunted rats. These results suggest that the accumulation of neutral amino acids in brain after portacaval shunt depends on the increased synthesis of glutamine in brain. **Key Words:** Portacaval anastomosis—Hyperammonemia—Brain glutamine—Large neutral amino acids—Methionine sulfoximine—Blood-brain amino acid transport. Rigotti P. et al. Methionine sulfoximine prevents the accumulation of large neutral amino acids in brain of portacaval-shunted rats. *J. Neurochem.* 44, 929–933 (1985).

After portacaval anastomosis (PCA) in rats, diversion of ammonia-rich portal blood into the systemic circulation results in a rise in blood ammonia concentrations to a level about twice normal. Brain concentrations of glutamine rise two- to three-fold by 4–6 weeks after PCA, presumably because increased flux of ammonia into brain stimulates the production of glutamine from ammonia and glutamic acid by the enzyme glutamine synthetase (EC 6.3.1.2) (Williams et al., 1972). The rise in brain glutamine concentration further suggests that the rate of removal of glutamine from brain does not match the increased rate of glutamine formation until a higher steady-state concentration of glutamine is reached. High glutamine concentrations in the CSF are a common finding in patients with liver cirrhosis and spontaneous portal-systemic circulatory shunting. CSF glutamine concentration has been shown to correlate reasonably well with the clinical grade of encephalopathy (Gilon et al., 1959; Hourani et al., 1971).

After PCA, the brain concentrations of several of the large neutral amino acids (LNAA), particularly phenylalanine, tyrosine, tryptophan, histidine, and

methionine, also increase (Curzon et al., 1975; James et al., 1978; Zanchin et al., 1979; Mans et al., 1982). This operation also has marked effects on the plasma concentrations of the various LNAA, resulting in decreased concentrations of leucine, isoleucine, valine, and threonine, and increased levels of phenylalanine, tyrosine, and histidine (James et al., 1976; Mans et al., 1982). However, altered competition among these plasma amino acids for blood-brain transport does not appear sufficient to account for the rise in brain LNAA concentrations (Bloxam and Curzon, 1978; James et al., 1979; Mans et al., 1982). Therefore, some other factor must also contribute to high brain LNAA levels after PCA.

One factor that has been suggested to contribute to high brain LNAA levels is an increased rate of uptake of these amino acid by brain after PCA. Blood-brain transport activity for several neutral amino acids has, in fact, been found to be higher in rats several weeks after PCA (James et al., 1978; Zanchin et al., 1979; Mans et al., 1982). Studies of amino acid transport in brain microvessels isolated from rats with a PCA have shown that the uptake

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Abbreviations used: LNAA, Large neutral amino acids; MSO, L-Methionine-*dl*-sulfoximine; PCA, Portacaval anastomosis.

of LNAA, but not of acidic or basic amino acids, is higher than normal (Cardelli-Cangiano et al., 1981). This observation suggests that the PCA operation may result in a change in the number or activity of the carriers that constitute the LNAA transport system of the blood-brain barrier. Studies of amino acid uptake by normal bovine brain capillaries have shown that the uptake of LNAA can be stimulated by preloading the microvessels either with glutamine or methionine, suggesting that a high brain concentration of LNAA could, in itself, increase the rate of unidirectional transport of other LNAA into the brain (Cangiano et al., 1983). It remains unclear, therefore, whether the increase in brain neutral amino acid uptake measured *in vivo* in rats after PCA or *in vitro* in brain microvessels isolated from such rats is a cause or an effect of high brain LNAA concentrations.

When normal rats (with an intact portal circulation) are infused with ammonium salts for 24 h, the brain concentrations of the LNAA and of glutamine rise in parallel (Gimmon et al., 1981; James et al., 1981). Jonung et al. (1984) observed that treatment of normal rats with L-methionine-*D*-sulfoximine (MSO) blocks the rise in the brain concentrations of glutamine and the LNAA caused by a 24-h ammonia infusion. In the present study, we measured plasma and brain amino acid levels in rats 2 weeks after PCA that were pretreated for 24 h with MSO in order to determine what effect lowering brain glutamine levels (while ammonia levels were still high) would have on the brain LNAA concentrations of rats with chronic portal-systemic shunts.

MATERIALS AND METHODS

Male Sprague-Dawley rats (300–350 g) underwent surgical creation of an end-to-side portacaval anastomosis under ether anesthesia as described by Lee and Fisher (1961) utilizing clean but not sterile surgical technique. Unoperated animals of the same age and weight were used as controls. Standard laboratory chow (#5001, Ralston Purina Co.) and water were available at all times. Rats were killed 13–15 days after surgery.

Twenty-four hours before decapitation, rats received an intraperitoneal injection either of MSO (Sigma Chemical Co., St. Louis, MO) at a dose of 150 mg/kg dissolved (50 mg/ml) in 0.15 mol/L NaCl, or of diluent alone. This dose of MSO caused no convulsions in any animal.

Amino acid analyses

Blood from the neck was collected in heparinized beakers. The plasma was separated by centrifugation and kept frozen at -72°C until analyzed. The brain was removed and divided sagittally into two hemispheres and frozen in liquid nitrogen. Plasma and brain amino acids were determined by a Beckman 121-MB amino acid analyzer with automatic integration. Plasma was deproteinized by mixing 0.5 ml of plasma with 1.5 ml of 5% (wt/vol) sulfosalicylic acid (pH 1.7) containing 133 nmol/ml of thienylalanine as internal standard. After vortex-mixing, the samples were centrifuged ($30,000 \times g$, 4°C)

and passed through a $0.45 \mu\text{m}$ Millipore filter. One-half brain from each rat was homogenized in three times its weight of 5% (wt/vol) sulfosalicylic acid (pH 1.4) containing 133 nmol/ml of thienylalanine. To quantitate glutamine accurately, the supernatant of the brain homogenate was further diluted 1:25 with 0.15 mol/L Li citrate buffer (pH 2.2) and analyzed in a special short analysis program.

Ammonia assay

Plasma and brain ammonia levels were measured in separate groups of rats killed in a way designed to minimize postmortem changes in ammonia concentrations. Under ether anesthesia, blood was drawn from the abdominal vena cava and transferred to chilled, evacuated tubes containing heparin. Plasma was separated by centrifugation ($2000 \times g$, 15 min, 4°C) and transferred to a stoppered tube. The skull was then exposed by a midline incision and a plastic funnel was positioned over the calvarium. The brain was frozen by pouring liquid N_2 into the funnel for 3 min. The rat was then decapitated and the head frozen in liquid N_2 .

Plasma for ammonia assay was deproteinized by addition to a tube containing an equal volume of frozen 0.5 mol/L HClO_4 to which an additional volume of ice-cold 0.5 mol/L HClO_4 was then added. After centrifugation ($10,000 \times g$, 15 min, 4°C) the supernatant was neutralized to pH 7.0–7.5 by addition of 2.0 mol/L KCHO_3 ; precipitated KClO_4 was removed by centrifugation ($10,000 \times g$, 15 min, 4°C). The frozen heads were warmed to -5°C and brain tissue (0.4–0.7 g) was removed while frozen, weighed, and transferred to tubes containing 2.0 ml of frozen 0.5 mol/L HClO_4 . An additional 2.0 ml of ice-cold 5 mmol/L EDTA was added to each tube and the mixture homogenized for 10 s (on ice) with a Polytron homogenizer (Brinkmann Instruments). Homogenates were further treated to yield neutralized extracts as described above.

Ammonia was measured using glutamate dehydrogenase (EC 1.4.1.3) in a reaction mixture containing in 1.0 ml 0.25 mol/L Tris buffer (pH 8.0), 2 mmol/L α -ketoglutarate, 0.16 mmol/L NADPH, and 4.3 units of glutamate dehydrogenase. Neutralized sample extract was added in amounts (0.1–0.5 ml) depending on expected ammonia content and, when necessary, ammonia-free water was added to bring the final volume to 1.0 ml. The reaction was initiated by addition of sample and the mixture was incubated at room temperature for 60 min at which time the absorbance at 340 nm was measured. A blank from which enzyme was omitted was prepared for each sample.

Statistical significance of differences was determined by analysis of variance followed by the Newman-Keuls test.

RESULTS

The PCA operation resulted in higher concentrations of ammonia both in plasma (Table 1) and brain (Table 2) compared with unoperated rats. Administration of MSO (150 mg/kg) had little additional effect on plasma ammonia levels in rats after PCA, but resulted in a marked increase in brain ammonia.

The effects of the PCA on plasma amino acids

TABLE 1. Plasma ammonia and amino acids in rats after portacaval anastomosis

	Controls	PCA	PCA + MSO
Ammonia ($\mu\text{mol/ml}$)	0.10 $\pm$ 0.02 ^a (6)	0.20 $\pm$ 0.01 ^a (6)	0.22 $\pm$ 0.02 (6)
Amino Acids (nmol/ml)			
Threonine	231 $\pm$ 14 (7)	138 $\pm$ 6 ^a (7)	220 $\pm$ 7 ^a (7)
Valine	199 $\pm$ 10	150 $\pm$ 10 ^a	183 $\pm$ 15 ^a
Methionine	56 $\pm$ 2	48 $\pm$ 2	72 $\pm$ 3 ^a
Isoleucine	93 $\pm$ 4 ^a	45 $\pm$ 4 ^a	73 $\pm$ 8 ^a
Leucine	169 $\pm$ 7 ^a	89 $\pm$ 6 ^a	134 $\pm$ 15 ^a
Tyrosine	75 $\pm$ 3 ^a	125 $\pm$ 8 ^a	120 $\pm$ 7
Phenylalanine	62 $\pm$ 2	120 $\pm$ 6 ^a	111 $\pm$ 6
Histidine	83 $\pm$ 4	99 $\pm$ 5	90 $\pm$ 5
Glutamine	650 $\pm$ 24 ^a	967 $\pm$ 70 ^a	289 $\pm$ 25 ^a
Alanine	458 $\pm$ 33	441 $\pm$ 22	745 $\pm$ 42 ^a
Lysine	473 $\pm$ 28	297 $\pm$ 14 ^a	483 $\pm$ 15 ^a
Arginine	206 $\pm$ 9 ^a	147 $\pm$ 11 ^a	164 $\pm$ 15

Results are given as means $\pm$ SEM with number of rats in parentheses.

^a Significantly different from controls, $p < 0.01$ at least.

^b Significantly different from PCA, $p < 0.01$ at least.

^c Significantly different from PCA + MSO, $p < 0.01$ at least.

(Table 1) were similar to those reported previously from this and other laboratories (James et al., 1976; Mans et al., 1982). Rats with a PCA had low plasma concentrations of threonine, the branched-chain amino acids, leucine, isoleucine and valine, lysine, and arginine, while plasma tyrosine, phenylalanine, and glutamine were increased compared with unoperated controls. MSO treatment resulted in significantly higher plasma concentrations of threonine, the branched-chain amino acids, lysine, and alanine as compared with rats with a PCA alone. The plasma concentration of glutamine was markedly lower in MSO-treated rats compared with the other two groups. MSO had no effect on plasma levels of tyrosine, phenylalanine, or histidine compared with untreated rats with PCA.

TABLE 2. Brain ammonia and amino acids in rats after portacaval anastomosis

	Controls	PCA	PCA + MSO
Ammonia ($\mu\text{mol/g}$)	0.21 $\pm$ 0.02 ^a (6)	0.38 $\pm$ 0.03 ^a (6)	0.99 $\pm$ 0.08 ^a (6)
Amino Acids (nmol/g)			
Threonine	515 $\pm$ 16 ^a (7)	578 $\pm$ 34 (7)	443 $\pm$ 11 ^a (7)
Valine	74 $\pm$ 4	87 $\pm$ 7	59 $\pm$ 3 ^a
Methionine	45 $\pm$ 1 ^a	60 $\pm$ 3 ^a	31 $\pm$ 4 ^a
Isoleucine	40 $\pm$ 1	33 $\pm$ 3 ^a	28 $\pm$ 1
Leucine	83 $\pm$ 3	87 $\pm$ 5	60 $\pm$ 3 ^a
Tyrosine	67 $\pm$ 3	276 $\pm$ 19 ^a	92 $\pm$ 7 ^a
Phenylalanine	42 $\pm$ 1 ^a	213 $\pm$ 10 ^a	72 $\pm$ 5 ^a
Histidine	69 $\pm$ 1	241 $\pm$ 18 ^a	81 $\pm$ 6 ^a
Glutamine	4824 $\pm$ 180 ^a	11493 $\pm$ 962 ^a	2644 $\pm$ 143 ^a
Alanine	611 $\pm$ 24 ^a	745 $\pm$ 19	1734 $\pm$ 84 ^a
Lysine	269 $\pm$ 14	247 $\pm$ 6	670 $\pm$ 23 ^a
Arginine	137 $\pm$ 4 ^a	128 $\pm$ 3	174 $\pm$ 10 ^a

Results are given as means $\pm$ SEM with number of rats in parentheses.

^a Significantly different from controls, $p < 0.01$ at least.

^b Significantly different from PCA, $p < 0.01$ at least.

^c Significantly different from PCA + MSO, $p < 0.01$ at least.

In brain, the PCA operation resulted in marked (four- to five-fold) elevations in tyrosine, phenylalanine, and histidine, as has been previously reported (James et al., 1978; Mans et al., 1982). Brain glutamine was increased to about twice the control values. MSO treatment resulted in significantly lower brain concentrations of threonine, valine, methionine, leucine, tyrosine, phenylalanine, histidine, and glutamine, and higher brain concentrations of alanine, lysine, and arginine, compared with untreated rats with PCA.

DISCUSSION

The high brain or CSF levels of various members of the group of LNAA that are observed in animals with a portacaval anastomosis have not yet been adequately explained. Bloxam and Curzon (1978) attempted to determine whether high brain levels of tryptophan after PCA could best be accounted for by changes in plasma-free tryptophan (that fraction of plasma tryptophan which is apparently unbound to albumin), plasma total tryptophan (bound plus free), or the altered plasma amino acid pattern after PCA and, hence, by changes in the competition tryptophan must face for blood-brain transport. Their results showed not only that no single one of these factors adequately explained the brain tryptophan elevations of PCA rats, but also that the ratio of free (or total) tryptophan in plasma to the sum of the plasma LNAA competitors correlated differently to brain tryptophan concentration in rats with PCA as compared with controls. Similar results were also demonstrated by these authors for plasma and brain tyrosine, the interpretation of which is not complicated by binding to albumin. These results, and similar data presented by James et al. (1979) for brain tyrosine, suggest that some factor other than plasma amino acid availability or competition for transport must be involved in elevating brain concentrations of the LNAA after PCA.

MSO treatment resulted in a marked increase in brain ammonia content, consistent with previous reports (Folbergrova et al., 1969; Cooper et al., 1979; Jonung et al., 1984). In the present study, it was anticipated that treatment with MSO would exert an effect on amino acid concentrations only in brain. In fact, as shown in Table 1, treatment with the glutamine synthetase inhibitor resulted in an increase in the plasma levels of some LNAA, in particular the branched-chain amino acids and threonine. The plasma level of methionine was also increased somewhat as compared with untreated PCA rats. It is possible that the rise in the plasma concentrations of the branched-chain amino acids in MSO-treated rats reflects the inhibition of glutamine synthesis in peripheral tissues, primarily skeletal muscle, of rats with PCA in which the

plasma ammonia concentration was increased to about twice normal (Table 1). Increased glutamine synthesis in muscle of PCA rats would be expected to result in increased utilization of glutamic acid in that tissue which might, in turn, stimulate increased transamination of the branched-chain amino acids with α -ketoglutarate. Inhibition of glutamine synthetase in muscle would be expected to reduce the utilization of glutamic acid to form glutamine and, thus, also to reduce the utilization of the branched-chain amino acids.

The rise in the concentrations of branched-chain amino acids in plasma after MSO treatment was not sufficient to restore their concentrations to control levels and, therefore, it is unlikely that the reductions in brain levels of phenylalanine, tyrosine, histidine, and threonine after MSO treatment can be accounted for entirely on the basis of a change in plasma ratios of competitors for blood-brain transport. In particular, it should be noted that the brain-to-plasma ratios of phenylalanine, tyrosine, and histidine in untreated PCA rats are greater than 2.0, whereas in MSO-treated rats these ratios were all less than 1.0. MSO treatment had no effect on the plasma concentrations of phenylalanine, tyrosine, and histidine.

The present results closely parallel those obtained previously in rats with intact portal circulation during infusion of ammonium salts (Jonung et al., 1984). In that study it was observed that MSO simultaneously blocked the rise in brain concentrations of glutamine and of other large neutral amino acids during 24-h infusion of ammonium salts at a rate sufficient to raise plasma ammonia to about 10 times normal levels. MSO did not affect plasma levels of the branched chain amino acids in that study. Taken together with the observations of Jonung et al. (1984), the present study suggests that the rise in brain levels of the neutral amino acids after PCA is due to hyperammonemia in those animals, but that this increase in brain amino acid levels is not due to ammonia alone. Mans et al. (1983) reported that the brain uptake of radiolabeled tryptophan injected in the carotid artery was increased in rats that were infused with ammonium acetate. This increased uptake was observed after 22 h, but not after 4 h, of infusion, although the brain concentration of glutamine was reported to be similarly elevated at both times. The authors concluded that the increased rate of tryptophan transport was not directly determined by glutamine. Since blood-brain transport was not directly measured in the present study, no conclusions can be drawn concerning the effect of MSO on LNAA transport in rats with a PCA.

MSO has been reported to reduce significantly the transport into brain of both tyrosine and valine in normal mice (Samuels and Fish, 1978; Samuels et al., 1978). These results were interpreted to sup-

port the involvement of the γ -glutamyl cycle in blood-brain transport of the LNAA, as previously proposed by Samuels (1977). Since the brain glutamine concentration was presumably decreased to about 75% of control levels under the conditions of these studies (Samuels et al., 1978), it is possible that the decreased uptake of tyrosine and valine in those studies simply reflected the lower brain glutamine concentration. Also, since MSO inhibits the activity of γ -glutamylcysteine synthetase (Richman et al., 1973), an enzyme in the γ -glutamyl cycle, it is possible that the present results reflect inhibition by MSO of the γ -glutamyl cycle and not simply inhibition of glutamine synthesis in brain.

Previous studies in this laboratory led to the proposal that high brain concentrations of glutamine after PCA might raise brain levels of the LNAA by a twofold mechanism involving (1) acceleration of exchange transport due to increased efflux of glutamine via the LNAA carrier in the blood-brain barrier and (2) decreased efflux of other LNAA from brain due to increased competition by glutamine for transport at the brain side of the blood-brain barrier (James et al., 1979). Regardless of whether glutamine efflux accelerates LNAA uptake by brain or inhibits LNAA efflux from brain, this hypothesis would predict that reducing brain glutamine in rats with a PCA would reduce brain LNAA to levels that were more consistent with the plasma LNAA competitor ratios. The present results are in accord with this prediction.

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Methionine Sulfoximine Prevents the Accumulation of Large Neutral Amino Acids in Brain of Hyperammonemic Rats

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Accumulation of neutral amino acids in the brain due to altered transport across the blood-brain barrier appears to be a consequence of portal-systemic shunting and hyperammonemia. It has been suggested that high brain concentrations of glutamine, a product of cerebral ammonia detoxification, accelerates the transport of other neutral amino acids from blood to brain. To test this hypothesis, normal rats were infused with ammonium salts with or without pretreatment with L-methionine-*dl*-sulfoximine (MSO), an inhibitor of glutamine synthesis. Pretreatment with MSO prevented most ammonium salt-induced changes in the concentrations of the neutral amino acids in brain, suggesting that hyperammonemia alters the transport of neutral amino acids across the blood-brain barrier by causing the brain glutamine level to rise.

INTRODUCTION

In conditions accompanied by hyperammonemia, such as chronic liver disease or after portacaval anastomosis, both in man and experimental animals, there is an accumulation of several of the large neutral amino acids (LNAA) in the CNS [3, 9, 14, 17]. Although in these conditions plasma levels of some of these amino acids may be somewhat higher than normal, the increase in brain is much greater than the increase in plasma.

Some of these amino acids, such as tyrosine, phenylalanine, tryptophan, or histidine, are precursors for neurotransmitters or related compounds. Since alterations in neurotransmitter metabolism may play a role in hepatic encephalopathy [1], it is of interest to learn why the LNAA reach such high brain levels in liver disease. In rats with a portacaval anastomosis, increased brain uptake of LNAA and decreased uptake of basic amino acids have been reported [3, 6, 17]. These observations suggest that the high brain concentrations of the LNAA may be related to altered transport across the blood-brain barrier.

Infusion of ammonium salts in normal rats results in a parallel rise in the brain concentrations of glutamine and the LNAA [2, 5]. Glutamine, an end product of ammonia detoxification in brain, probably is also transported by the L-system carrier in the blood-brain barrier [8, 11]. These observations suggest that high levels of glutamine in the brain might alter the transport of LNAA across the blood-brain barrier [4]. To test this suggestion, we measured brain amino acid levels in normal rats infused with ammonia for 24 hr and in rats pretreated with L-methionine-*dl*-sulfoximine (MSO), an inhibitor of glutamine synthetase.

MATERIALS AND METHODS

Male Sprague-Dawley rats (270-340 g) underwent placement of a Silastic catheter in the external jugular vein under ether anesthesia. The catheter was protected by a flexible metal sheath attached to a swivel. Rats were infused for 24 hr either with 0.15 M NaCl or with a solution containing 0.2 M ammonium acetate and 0.2 M ammonium bicarbonate, pH 7.4. Either infusion was delivered at a rate of 0.7 ml/hr/100 g body wt. One group of rats re-

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ceived MSO (150 mg/kg, ip) at a concentration of 50 mg/ml in 0.15 M NaCl 2 hr before beginning infusion of ammonium salts.

Amino acid analyses. After 24 hr of infusion, the rats were decapitated and blood from the neck was collected into heparinized beakers. The plasma was separated by centrifugation and kept frozen at -72°C until analyzed. The brain was removed and divided sagittally into two hemispheres and frozen in liquid nitrogen. Plasma and brain amino acids were determined by a Beckman 121-MB amino acid analyzer with automatic integration. Plasma was deproteinized by mixing 0.5 ml of plasma with 1.5 ml of 5% (wt/vol) sulfosalicylic acid, pH 1.7, containing 133 nmole/ml of thienylalanine as internal standard. After vortexing, the samples were centrifuged (30,000g, 4°C) and passed through a 0.45- μm Millipore filter. The brain was homogenized in three times its weight of 5% (wt/vol) sulfosalicylic acid, pH 1.4, containing 133 nmole/ml of thienylalanine as above. Fifty microliters of the supernatant of each brain homogenate was analyzed. To accurately quantitate glutamine, glutamate, and aspartate, the supernatant of the brain homogenate was further diluted 1:25 with 0.15 M Li citrate buffer, pH 2.2, and analyzed using a special short analysis program.

Ammonia assay. Plasma and brain ammonia levels were measured in separate groups of rats, treated as before but killed in a way to minimize postmortem changes in ammonia levels. Under ether anesthesia blood was drawn from the abdominal vena cava and transferred to chilled, evacuated tubes containing heparin. The ammonia-infused rats were anesthetized while the infusion was in progress; blood was withdrawn within 2 min of stopping the infusion. Plasma was separated by centrifugation (2000g, 15 min, 4°C) and transferred to a stoppered tube. The skull was then exposed by a midline incision and a plastic funnel was positioned over the calvarium. The brain was frozen *in situ* by pouring liquid N_2 into the funnel for 3 min. The rat was then decapitated and the head frozen in liquid N_2 .

Plasma was deproteinized by addition to a

tube containing an equal volume of frozen 0.5 M HClO_4 to which an additional volume of ice-cold 0.5 M HClO_4 was then added. After centrifugation (10,000g, 15 min, 4°C) the supernatant was neutralized to pH 7.0–7.5 by addition of 2.0 M KHCO_3 ; precipitated KClO_4 was removed by centrifugation (10,000g, 15 min, 4°C). The frozen heads were warmed to -5°C and brain tissue (0.4–0.7 g) was removed while frozen, weighed, and transferred to tubes containing 2.0 ml of frozen 0.5 mole/liter HClO_4 . An additional 2.0 ml of ice-cold 5 mM EDTA was added to each tube and the mixture was homogenized for 10 sec (on ice) with a Polytron homogenizer (Brinkman Instruments). Homogenates were further treated to yield neutralized extracts as described above.

Ammonia was measured using glutamate dehydrogenase in a reaction mixture containing in 1.0 ml 0.25 M Tris buffer, pH 8.0, 2 mM α -ketoglutarate, 0.16 mmole/liter NADPH, and 4.3 units of glutamate dehydrogenase. Neutralized sample extract was added in amounts (0.1–0.5 ml) depending on expected ammonia content and, when necessary, deionized water was added to bring the final volume to 1.0 ml. The reaction was initiated by addition of sample and the mixture was incubated at room temperature for 60 min at which time the absorbance at 340 nm was measured. A blank from which enzyme was omitted was prepared for each sample.

Statistical significance of differences was determined by analysis of variance followed by the Newman-Keuls test.

RESULTS

Rats infused with ammonium salts had plasma and brain ammonia levels which were much higher than those in rats infused with saline (Table 1). Administration of MSO before infusion of ammonium salts resulted in a greater increase in ammonia levels in brain than in plasma. The effect of the various treatments on plasma amino acid levels (Table 2) was slight. Compared to saline-infused controls, rats receiving ammonium salts without MSO pretreatment had high plasma concen-

TABLE 1
CONCENTRATIONS OF AMMONIA IN PLASMA AND BRAIN
OF RATS AFTER INFUSION OF SALINE OR
AMMONIUM SALTS FOR 24 hr

	Saline (6)	Ammonium salts (6)	MSO, ammonium salts (6)
Plasma (μ mole/ml)	0.10 $\pm$ 0.02	0.99 $\pm$ 0.13	1.29 $\pm$ 0.19
Brain (μ mole/g)	0.21 $\pm$ 0.02	1.61 $\pm$ 0.22	2.75 $\pm$ 0.21

Note. Results are given as mean $\pm$ SEM for the number of rats shown in parentheses.

trations of glutamine. In contrast, rats receiving both MSO and ammonium salts had low plasma glutamine levels, presumably reflecting inhibition of glutamine synthetase in peripheral tissues. Plasma concentrations of lysine and arginine were decreased compared to control rats in both groups of ammonia-infused rats.

In the brains of rats infused with ammonium salts without MSO pretreatment, the

concentrations of glutamine and the LNAA were significantly greater than in the controls (Table 3). In rats pretreated with MSO, however, brain levels of glutamine and the LNAA were only slightly higher than in the controls and significantly lower than in the rats receiving only ammonium salts. Compared to rats not receiving MSO pretreatment, MSO-treated rats had higher brain concentrations of the basic amino acids, lysine and arginine.

DISCUSSION

The rise in brain glutamine following infusion of ammonium salts probably reflects increased activity of glutamine synthetase due to high brain concentrations of ammonia. Pretreatment with MSO blocked the ammonia-induced rise in brain glutamine. Brain ammonia levels were higher in the ammonia-infused rats pretreated with MSO, as was previously observed by Warren and Scheuiker in 1964 [16]. Therefore, the increase in brain levels of the LNAA during infusion of ammonium salts would seem to be related to the rise in brain glutamine concentration and not a direct effect of a high brain ammonia concentration per se.

Conceivably, high levels of ammonia in brain might increase free amino acid concentrations by inhibiting protein synthesis or accelerating protein breakdown. It is difficult to explain how MSO could prevent this hypothetical effect of ammonia on brain protein metabolism or how the effect could be limited to a few amino acids. The concentrations of glutamine and the LNAA might be simultaneously increased in brain due to increased synthesis of the LNAA via transamination of their corresponding α -keto acids (supplied from the blood) with glutamine to yield the LNAA and α -keto glutaramate, the corresponding α -keto acid of glutamine which has been reported to be elevated in the CSF of patients in hepatic coma [15]. This explanation seems unlikely because threonine and histidine cannot be formed by transamination reactions, yet these two amino acids were both elevated by ammonia infusion. The mecha-

TABLE 2
CONCENTRATIONS OF AMINO ACIDS IN PLASMA
OF RATS AFTER INFUSION OF SALINE OR
AMMONIUM SALTS FOR 24 hr

Amino acids (nmole/ml)	Saline (6)	Ammonium salts (6)	MSO, ammonium salts (6)
Tryptophan	84 $\pm$ 5	90 $\pm$ 8	83 $\pm$ 5
Phenylalanine	67 $\pm$ 4	102 $\pm$ 3 ^a	79 $\pm$ 7 ^a
Tyrosine	68 $\pm$ 4	68 $\pm$ 7	53 $\pm$ 4
Methionine	55 $\pm$ 2	55 $\pm$ 11	51 $\pm$ 2
Histidine	65 $\pm$ 3	71 $\pm$ 1	66 $\pm$ 1
Leucine	138 $\pm$ 11	138 $\pm$ 12	132 $\pm$ 12
Isoleucine	77 $\pm$ 6	75 $\pm$ 7	76 $\pm$ 9
Valine	200 $\pm$ 13	163 $\pm$ 15	162 $\pm$ 14
Threonine	186 $\pm$ 11	161 $\pm$ 15	165 $\pm$ 15
Glutamine	900 $\pm$ 41	1445 $\pm$ 252	385 $\pm$ 30 ^b
Lysine	407 $\pm$ 15	346 $\pm$ 20 ^a	306 $\pm$ 23
Arginine	191 $\pm$ 10	139 $\pm$ 15 ^a	132 $\pm$ 9

Note. Results are given as mean $\pm$ SEM for the number of rats shown in parentheses.

^a Significantly different from concentration after saline infusion; $P < 0.05$ at least.

^b Significantly different from concentration after NH_3 infusion without MSO; $P < 0.05$ at least.

TABLE 3
CONCENTRATIONS OF AMINO ACIDS IN BRAIN OF RATS AFTER INFUSION WITH
SALINE OR AMMONIUM SALTS FOR 24 hr

Amino acids (nmole/g)	Saline (6)	Ammonium salts (6)	MSO, ammonium salts (6)
Tryptophan	18 ± 1	43 ± 5 ^a	26 ± 3 ^b
Phenylalanine	52 ± 4	230 ± 17 ^a	70 ± 3 ^b
Tyrosine	71 ± 10	164 ± 8 ^a	60 ± 4 ^b
Methionine	53 ± 3	97 ± 12 ^a	44 ± 3 ^b
Histidine	65 ± 2	213 ± 14 ^a	88 ± 6 ^b
Leucine	69 ± 5	170 ± 15 ^a	88 ± 4 ^b
Isoleucine	37 ± 4	69 ± 6 ^a	47 ± 3 ^b
Valine	72 ± 3	140 ± 5 ^a	80 ± 3 ^b
Threonine	461 ± 17	925 ± 112 ^a	453 ± 27 ^b
Glutamine	5530 ± 240	27400 ± 1200 ^a	6370 ± 270 ^b
Lysine	248 ± 18	335 ± 7 ^a	586 ± 29 ^b
Arginine	131 ± 2	136 ± 16	171 ± 3 ^b

Note. Results are given as mean ± SEM for the number of rats shown in parentheses.

^aSignificantly different from concentration after saline infusion; $P < 0.05$ at least.

^bSignificantly different from concentration after NH₄ infusion without MSO; $P < 0.05$ at least.

nism whereby high brain glutamine concentrations might lead to increased brain levels of the LNAA, but not of other amino acids, would seem to involve some aspect of blood-brain transport of the LNAA.

The LNAA share a common transport system for entry into brain [7, 12]. Glutamine apparently has a low affinity for this transport system, since the brain uptake index (corrected for label retained in brain vasculature) of a tracer amount of [¹⁴C]glutamine relative to ³H₂O is only about 5.0% [8]. The low uptake of glutamine was reported to be reduced in the presence of unlabeled phenylalanine, suggesting that glutamine and phenylalanine compete for the same transport system [8]. High levels of unlabeled glutamine (>2 mM) decrease the brain uptake index (BUI) of tracer amounts of [¹⁴C]phenylalanine (this laboratory, unpublished observations). If the transport of the LNAA from brain to blood is mediated by a system with the same properties as the system that mediates blood-to-brain transport, then it is possible that a brain concentration of glutamine in excess of 20 mmole/g could significantly impede the efflux of the LNAA from brain.

The concentration gradient of glutamine (and the other LNAA) from brain to blood may also "drive" exchange transport of LNAA. This effect, generally referred to as transacceleration, may be the cause of the increased unidirectional (blood-to-brain) transport of the LNAA which has been observed in rats after portacaval anastomosis. Exchange of brain glutamine for circulating LNAA has been specifically proposed as a potential function of the gamma-glutamyl cycle [13]. Gamma-glutamyl transpeptidase is present in cerebral capillaries [10], but it is not known at present whether the gamma-glutamyl cycle is, in fact, involved in blood-brain amino acid transport. Although the present results do not permit us to select among possible transport mechanisms, a potential role for brain glutamine in modifying blood-brain transport of the LNAA in conditions of hyperammonemia would appear to be consistent both with established observations and proposed hypotheses.

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EFFECT OF HYPERAMMONEMIA AND METHIONINE SULFOXIMINE ON THE
KINETIC PARAMETERS OF BLOOD-BRAIN TRANSPORT OF LEUCINE AND PHENYLALANINE

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Abbreviations Used: PCA: portacaval anastomosis
LNAA: large neutral amino acids
MSO: L-methionine-dl-sulfoximine
HRP: horseradish peroxidase

ABSTRACT

The activity of the blood-brain neutral amino acid transport system is increased in rats infused with ammonium salts or rendered hyperammonemic by a portacaval anastomosis. This effect may be due to a direct action of ammonia or to some metabolic consequence of high ammonia levels, such as increased brain glutamine synthesis. To test these possibilities we evaluated the kinetic parameters of blood-brain transport of leucine and phenylalanine in control rats, in rats after continuous 24 h infusion of ammonium salts ($\text{NH}_4^+ = 2.5 \text{ mmol}\cdot\text{kg}^{-1}\cdot\text{hr}^{-1}$) and in rats treated with methionine sulfoximine, an inhibitor of glutamine synthetase, before infusion of ammonium salts. In ammonia-infused rats without methionine sulfoximine treatment, the K_d and V_{max} of phenylalanine transport were increased, respectively, about 170% and 80% compared to controls, while the K_m and V_{max} of leucine transport were increased, respectively, about 100% and 200%. Administration of methionine sulfoximine before ammonia-infusion inhibited glutamine synthesis and prevented these changes in transport of leucine and phenylalanine. These results suggest that the ammonia-induced increase in the activity of transport of large neutral amino acids across the blood-brain barrier requires glutamine synthesis in brain, and is not a direct effect of ammonia.

Key Words: Ammonia; Methionine sulfoximine; Amino acid transport; Blood-brain barrier; Glutamine synthesis.

Running Title: Ammonia and blood-brain transport kinetics

Diversion of ammonia-rich portal blood into the systemic circulation results in general hyperammonemia. This alteration in normal blood flow can occur spontaneously, when flow through the liver is blocked by liver cirrhosis, or can be experimentally reproduced by surgical creation of a portacaval anastomosis. After portacaval anastomosis, the transport of the large neutral amino acids (LNAA) into brain is increased (James et al., 1978; Zanchin et al., 1979; Mans et al., 1982). Also, the concentrations in brain of glutamine and of some of the LNAA are increased several-fold after portacaval anastomosis (Williams et al., 1972; Curzon et al., 1975; James et al., 1978). The relationship between increased blood-brain transport of the LNAA and increased brain concentrations of glutamine and other LNAA has not yet been established.

Intravenous infusion of ammonium salts or intraperitoneal urease injection in rats with intact portal circulation also result in increased levels of glutamine and LNAA in brain, while having little effect on LNAA concentrations in plasma (James et al., 1980; Gimmon et al., 1981; Bachmann and Colombo, 1983; Jonung et al., 1984). The rise in LNAA concentrations apparently involves their transport across the blood-brain barrier, since raising the blood levels of the branched chain amino acids (leucine, isoleucine, valine) in ammonia-infused rats lowers the brain levels of the other LNAA, which compete for the same blood-brain transport system (Gimmon et al., 1981; James et al., 1981). Moreover, pretreatment with L-methionine-dl-sulfoximine (MSO) before ammonia infusion blocks not only the increase in brain glutamine concentration but also the increase in the brain concentrations of the other LNAA (Jonung et al., 1984), suggesting a

relationship between the rise in brain glutamine and the rise in brain levels of the other LNAA after ammonia infusion.

The relationship between hyperammonemia and blood-brain LNAA transport activity has not been studied in detail. Mans et al. (1983) measured the uptake of a trace concentration of radiolabeled tryptophan 5 s after injection in the carotid artery of rats infused for various times with ammonium acetate. In that study, brain uptake of tryptophan was significantly increased after 22 h of infusion, when brain glutamine was elevated about 3-fold, but not after only 4 h of infusion, when the brain glutamine concentration was nearly as high as at 22 h. In the present studies, we determined the effect of infusion of ammonium-salts and pretreatment with MSO on the kinetics of transport of two of the LNAA, leucine and phenylalanine, across the blood-brain barrier.

MATERIALS AND METHODS

Materials:

L-[U- ^{14}C] phenylalanine (450 mCi/mmol), L-[U- ^{14}C] leucine (300 mCi/mmol), [U- ^{14}C] sucrose (360 mCi/mmol) and n -[1- ^{14}C] butanol (0.9 mCi/mmol) were obtained from New England Nuclear, Boston, MA. L-methionine-d $_3$ -sulfoximine, L-glutamic dehydrogenase (EC 1.4.1.3) and horseradish peroxidase (EC 1.11.1.7) were obtained from Sigma Chemical Co., St. Louis, MO.

Infusion of Ammonium Salts:

male Sprague-Dawley rats (300-350 g, were infused at a rate of 6-7

$\text{ml}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ with a 0.4 mol/l solution of ammonium salts (0.2 mol/l ammonium acetate, 0.2 mol/l ammonium bicarbonate, pH 7.4) which had previously been passed through an 0.45 micron filter (Millipore Corp.). The infusion was delivered by a Holter pump (Extracorporeal Co.) via a silastic catheter in the right jugular vein which exited the rat through the skin of the mid-scapular area. The catheter was protected by a metal spring attached to an infusion swivel. Water, but not food, was available during the infusions. The infusion was continued for 22-24 h, which was previously determined to result in a 4-to-5-fold increase in brain glutamine concentration (Gimmon et al., 1981). Normal plasma osmolarity was maintained. MSO (150 mg/kg) was dissolved (50 mg/ml) in 0.15 mol/l NaCl and injected intraperitoneally 30 min before beginning infusion. This dose of MSO did not cause convulsions. Food was withdrawn from control rats, which were otherwise untreated.

Ammonia Assay:

Plasma and brain ammonia concentrations were determined in separate groups of rats which were not used for transport measurements, but which were otherwise treated identically before sacrifice. These rats were killed as previously described (Jonung et al., 1984) to minimize post-mortem increases in brain ammonia. Briefly, rats were anesthetized with diethyl ether and blood drawn from the abdominal aorta. The skull was then exposed and the brain frozen in situ by pouring liquid N_2 for a period of 3 min into a plastic funnel fixed to the head. Plasma and brain samples were deproteinized in 0.5 mol/l HClO_4 and assayed for ammonia using the glutamic dehydrogenase reaction as previously described (Jonung et al., 1984).

Brain Glutamine Analysis:

For subsequent analysis of glutamine, a portion of the frontal cortex from each rat receiving ammonium salts in the amino acid transport studies was dissected from the side of brain contralateral to the carotid injection, described below. The brain tissue was frozen on solid CO₂ and stored at -72°C until analysis. Glutamine was quantified using a Beckman 121-MB amino acid analyzer after homogenization of the tissue in 3 volumes (vol/wt) of 5% (wt/vol) sulfosalicylic acid. After centrifugation the supernate was further diluted 1:25 with 0.15 mol/l Li citrate buffer, pH 2.2, before analysis. On the basis of this analysis, rats receiving ammonium salts, but not MSO, and which had a brain glutamine concentration below 15 mmol/kg were excluded from the transport analysis. Similarly, rats receiving MSO but with a brain glutamine concentration above 6 mmol/kg were excluded. On this basis, data from 5 rats out of a total of 170 were discarded.

Carotid Injection Technique:

The brain uptake index was measured by the method of Oldendorf (1971). Rats were anesthetized with sodium pentobarbital (45-50 mg/kg) about 15 min before rapid injection in a carotid artery of 0.2 ml of a buffered Ringer's solution (pH 7.4, 10mM HEPES) containing ¹⁴C-butanol, ¹⁴C-amino acid or ¹⁴C-sucrose (1-2 µCi/ml) and [³H]-water (5-10 µCi/ml). A piece of frontal cortex (0.15-0.25 g) was dissected from the side of brain ipsilateral to the injection and solubilized in tightly-closed tubes with 1.0 ml TS-1 tissue solubilizer (Research Products International, Mount Prospect, IL) at 50°C for 3-4 h. Samples were counted in an LKB Model 1217 liquid scintillation

counter using external standardization and dual label correction. The brain uptake index was calculated as:

$$\text{BUI} = \frac{{}^{14}\text{C}/{}^3\text{H} \text{ (dpm in brain)}}{{}^{14}\text{C}/{}^3\text{H} \text{ (dpm in injection solution)}} \times 100$$

Calculation of Transport Parameters:

The rate of cerebral blood flow (F) and extraction of water ($E_{\text{H}_2\text{O}}$) were determined exactly as described by Pardridge et al. (1982) with the difference that we followed the butanol washout at 0.25, 1.0, and 2.0 min after carotid injection of [${}^{14}\text{C}$]-butanol and [${}^3\text{H}$]-water, omitting the four-minute point to avoid possible error due to recirculation (Bradbury et al., 1975). Since the treatments involved may have influenced F, this parameter was determined separately for each treatment group.

The K_m and V_{max} of the saturable component of transport and the K_d or constant of non-saturable transport were determined from a nonlinear regression analysis of the BUI data as described by Pardridge et al. (1982).

The unidirectional clearance (V/C) is defined as follows:

$$\frac{V}{C} = \frac{V_{\text{max}}}{K_m + \bar{C}} + K_d$$

$$\frac{V}{C} = EF$$

$$E = (\text{BUI}_{\text{amino acid}} - \text{BUI}_{\text{sucrose}}) (E_{\text{H}_2\text{O}})/100$$

where $\bar{C}$ = the mean capillary concentration of amino acid; V = unidirectional influx; E = extraction fraction of unidirectional influx; F = cerebral blood

flow; $BUI_{\text{amino acid}}$ and BUI_{sucrose} are the uptake indices relative to $[^3\text{H}]$ -water of amino acid or sucrose, respectively; and $E_{\text{H}_2\text{O}}$ = the extraction of $[^3\text{H}]$ -water relative to $[^{14}\text{C}]$ -butanol reference at 15 s after injection. Subtraction of BUI_{sucrose} provided a correction for residual radioactivity remaining in the brain vasculature. The mean capillary concentration, $\bar{C}$, was calculated from the arterial or injection solution concentration (C_a) as follows:

$$\bar{C} = \frac{E}{-\ln(1-E)} \cdot C_a$$

High values of E , as observed in ammonia-infused rats, require this correction for change in concentration due to uptake during passage through the capillary (Pardridge and Mietus, 1982).

Statistical Analysis of Data:

Non-linear regression analysis was done by the University of Cincinnati Computer Center's Amdahl 470 computer using subroutine P3R of BMDP (Biomedical Computer Programs P Series) developed at Health Sciences Computing Facility, UCLA (Los Angeles, CA). Testing of the statistical significance of differences in individual parameters was carried out only if a significant overall F-statistic for the difference between the regression equations themselves was obtained. Statistical significance of differences between values for individual transport parameters was evaluated by Student's t-test. In order to gather uptake data over a sufficiently wide range of values to permit confident calculation of parameters of both saturable and non-saturable transport, the concentration of unlabeled amino

acid in the injection solution was varied from 0.004-0.008 mM to 8 mM (Table 3).

Electron Microscopy Studies of the Integrity of the Blood-Brain Barrier:

Rats were injected with horseradish peroxidase (HRP) as described by Laursen and Westergaard (1977). Under pentobarbital anesthesia, brains were fixed in situ by a 15-min vascular perfusion with a solution of 2% (wt/vol) paraformaldehyde and 2.5% (vol/vol) glutaraldehyde in 0.08 M sodium cacodylate buffer at pH 7.3. The rats were then decapitated, the brain removed and placed in a solution of 4% (wt/vol) paraformaldehyde, 5% (vol/vol) glutaraldehyde in 0.08 M sodium cacodylate buffer, pH 7.3, and allowed to fix from four hours to overnight at 4°C. The fixed brain were cut into frontal slices approximately 0.5 mm thick with a Stadie-Riggs tissue slicer. Areas from the cerebral cortex, cerebellum, basal ganglia (corpus striatum) and brain stem were dissected out of the slices and cut into small blocks 0.5 to 1 mm on a side. Tissue blocks were rinsed overnight in 0.1 M cacodylate buffer. Next day, the tissue blocks were rinsed in Tris buffer (0.05 M, pH 7.6) and placed in 30 ml of 0.05 M Tris HCl (pH 7.6) containing 1 mg/ml diaminobenzidine with 0.03% hydrogen peroxide added at 4°C with constant agitation for 3 h. All brain samples were postfixed with 2% (wt/vol) osmium tetroxide in 0.1 M sodium cacodylate buffer for 3 h at room temperature, dehydrated in ethyl alcohol and embedded in Spurr epoxy resin. Sections were examined both unstained and stained with uranyl acetate followed by lead citrate.

RESULTS

Infusion of ammonium salts raised the brain ammonia concentration (Table 1) about 8-fold; pretreatment with MSO resulted in still higher brain ammonia concentration after ammonia infusion, confirming previous observations (Jonung et al., 1984). Brain glutamine in rats receiving only ammonium salts was increased about 4-fold over controls (Table 1). Rats receiving MSO pretreatment before ammonia infusion had brain glutamine concentrations which were slightly lower than in control.

The BUI of [^3H]-water with respect to the [^{14}C]-butanol reference and the fractional extraction of water ($E_{\text{H}_2\text{O}}$) were similar in all groups (Table 2). The BUI of sucrose relative to [^3H]-water, which presumably represents label retained in the cerebral vasculature, was significantly increased in both ammonia-infused groups compared to controls. These values were subtracted from the amino acid BUI values of the corresponding group before computing the transport parameters. The rates of cerebral blood flow obtained in all three groups were in good agreement with those reported by others for the pentobarbital-anesthetized rat (Gjedde and Rasmussen, 1980).

The BUI values, less the $\text{BUI}_{\text{sucrose}}$, for the uptake of phenylalanine and leucine at different concentrations of competing unlabeled amino acids are shown in Table 3. The transformation of these data to a $V/\bar{C}$ vs $\bar{C}$ plot is shown in Figure 1, from which the data from MSO-pretreated rats has been omitted for clarity. Although mean values at each concentration point are shown in Table 3 and Figure 1, the individual values of $V/\bar{C}$ and $\bar{C}$, as calculated for each rat, were used for the non-linear regression analysis to

determine the transport parameters.

The K_m of transport of phenylalanine was similar in all three groups (Table 4), while the V_{max} of transport was significantly higher in the rats receiving only ammonium salts as compared with both of the other groups. The transport K_d or constant of apparently non-saturable transport of phenylalanine was also increased significantly in the ammonia-infused rats as compared with the other two groups (Table 4).

With regard to leucine transport, both the K_m and V_{max} of uptake were significantly greater in the rats receiving only ammonium salts as compared with control or MSO-pretreated rats. MSO-treated rats had lower values of K_m , V_{max} and K_d for leucine transport compared to the untreated control animals, although these differences were not statistically significant. In contrast to the K_d of phenylalanine uptake in rats receiving only ammonium salts, the K_d of leucine transport was not significantly increased by the same treatment.

Microscopy Studies:

Control tissues: Tissue from the control brains had HRP reaction product present along the luminal border of the endothelial cells of capillaries. No reaction product was seen in the subendothelial basement membrane, interendothelial space, or in the adjacent neuropil. Overall, the uptake of HRP by the neural vasculature was slight. No areas of perivascular swelling were observed. These findings were consistent throughout all areas of the brains studied.

Experimental tissues: Brains from the ammonia-treated rats had wide

electron-translucent areas around the capillaries and venules (Figs. 2,3). These areas of perivascular dilation, which were easily visible in sections prepared for light microscopy, ranged in size from small intracellular swellings confined within astrocyte end-feet to large spaces extending several microns into the adjacent neuropil and often completely surrounding the adjacent vessel. In rare instances, perivascular swelling appeared to cause compression of the capillary (Figs. 3B-3C). Large dilated areas contained cellular debris in the form of trilaminar membrane fragments, precipitated protein granules, mitochondria and necrotic nuclei. Interendothelial tight junctions were intact and a normal basement membrane was maintained around adjacent capillaries (Figs. 2C-2D). Pinocytotic vesicles were not found in cerebral cortex and rarely seen elsewhere; Fig. 2C shows a membrane-bound vesicle, apparently containing HRP reaction product observed in brain stem.

Isolated areas of swelling with no vascular contact were sometimes seen in sections. It was assumed that these were extensions of areas around vessels located out of the plane of the section. As in the control tissues, uptake of HRP was slight and was confined to a thin layer of reaction product on the luminal surface of endothelial cells and in small scattered caveolae and pinocytotic vesicles. No reaction product was seen in the endothelial basement membrane, interendothelial space, or in the perivascular dilations, emphasizing the integrity of the cerebral capillaries (Figs. 2 and 3). Findings in the group of rats receiving MSG pretreatment and ammonium salts were similar to those in the group of rats receiving ammonium salts alone.

DISCUSSION

Altered blood-brain transport of the LNAA has been described in animal models of liver disease and portal-systemic shunting of the blood (James et al., 1978; Zanchin et al., 1979; Mans et al., 1982) and also in patients with liver cirrhosis (Sato et al., 1981). The present studies were undertaken to examine which of the kinetic parameters of blood-brain transport were affected by acute hyperammonemia in order to gain insight into the mechanisms of such alterations. Increased blood-brain transport of HRP has been reported in rats following portacaval anastomosis (Laursen and Westergaard, 1977), perhaps due to increased pinocytotic transport activity in cerebral capillaries. Since increased pinocytotic transport could conceivably contribute to blood-brain transport in ammonia-infused rats, evidence for brain uptake of HRP was sought in the present studies. We also looked for evidence of physical disruption of the blood-brain barrier because the swelling of glial astrocytes, which is known to occur in hyperammonemic animals (Zamora et al., 1973), might affect the capillary endothelial cells with which glial processes are in close contact. Finally, since it has been reported that MSO reduces the abnormal accumulation of LNAA in brains both of ammonia-infused rats (Jonung et al., 1984) and of portacaval-shunted rats (Rigotti et al., 1984), it was of interest in the present studies to examine the effect of MSO on transport kinetic parameters.

The relationship between ammonia-induced ultrastructural changes in the blood-brain barrier and the observed changes in kinetic parameters should be

considered. Watery swelling of astrocytes is commonly observed in many species following treatments which cause hyperammonemia either by liver damage, portal-systemic shunting of the circulation, or administration of ammonia itself (for review, see Diemer, 1978). To our knowledge, present studies provide the first evidence that widespread glial swelling occurs after only 24 h of hyperammonemia. In the present studies, neither leakage of HRP into the interstitial space of brain nor opening of endothelial tight-junctions could be demonstrated. Since MSO pre-treatment was associated with a return toward normal of the values of the transport kinetic parameters but was not associated with reduced astrocyte swelling, it seems unlikely that the changes in transport kinetic parameters and in glial ultrastructure are related to one another. Also, the lack of effect of MSO on astrocyte swelling indicates that an enhanced rate of glutamine synthesis, per se, is not the cause of such swelling.

The observed values of K_m , V_{max} and K_d for both leucine and phenylalanine transport in the control rats compared reasonably well with those previously reported for adult rats by Pardridge and Mietus (1982). The differences between the present values and those of Pardridge and Mietus (1982) may reflect technical differences: the values obtained in the present study were obtained using ten different concentrations ranging from trace to 8 mM, whereas those obtained by Pardridge and Mietus (1982) were based on five concentrations ranging from trace to 4 mM (Pardridge and Oldendorf, 1974). Infusion of ammonium salts without MSO was associated with a significant increase in V_{max} for both amino acids tested, whereas pretreatment with MSO prevented those increases (Table A). Since brain LNAA

concentrations are increased in ammonia-infused rats and since MSO blocks this rise (Jonung et al., 1984), the present results suggest that increased blood-brain LNAA transport activity may contribute to the rise in brain LNAA concentrations in hyperammonemic rats.

It is also possible that the rise in transport $V_{\max}$ for phenylalanine and leucine reflects accelerated exchange transport, secondary to increased concentrations of glutamine and the other LNAA at the antiluminal capillary surface, and hence within capillary endothelial cells. The uptake of tyrosine or leucine in vitro by isolated brain microvessels can be stimulated markedly by preloading the microvessels with either glutamine or another LNAA, methionine; this acceleration of uptake declines rapidly after the microvessels are transferred to amino acid-free medium, in parallel with the efflux of the preloaded amino acid from the microvessels (Cangiano et al., 1983). Although the route of uptake in the studies of Cangiano et al. (1983) was presumably across the antiluminal membrane, rather than the luminal membrane, those studies suggested that the rate of exchange transport of LNAA could be accelerated by a high concentration of glutamine and other LNAA within capillary endothelial cells. Therefore, the increase in transport $V_{\max}$ in the present studies may be the result, rather than the cause, of high brain LNAA levels. If that is the case, then the rise in brain LNAA after ammonia infusion must be explained by some factor other than increased influx. This factor could be either a decreased rate of efflux of LNAA from brain or an increase of free amino acids in brain due to accelerated proteolysis or to reduced protein synthesis. We favor the former of these two explanations since, if ammonia disrupted protein

metabolism in brain, it would be difficult to explain how MSO could block that disruption.

Jonung et al. (1984) previously reported that MSO prevented the accumulation of glutamine in brain of ammonia-infused rats; the present studies confirm this observation. Since glutamine is transported by the blood-brain LNAA transport system (Oldendorf and Szabo, 1976; Pardridge and Mietus, 1982), Jonung et al. (1984) argued that high brain glutamine levels could lead to significant inhibition of the efflux of other LNAA from brain. Therefore, the present results are also consistent with the hypothesis that MSO prevents rises in transport V_{max} by inhibiting brain glutamine synthesis and thus the accumulation of glutamine and other LNAA in brain after ammonia infusion.

Ammonia infusion was also associated with a marked increase in the transport K_d of phenylalanine and a smaller rise in the transport K_d of leucine (Table 4), although the latter change did not reach statistical significance. The K_d may represent either a "diffusional" uptake, transport by another system with very high K_m , or both. The transport K_d 's for the LNAA are markedly higher in newborn rabbits than in adult rats (Pardridge and Mietus, 1982), and an approximately 2-fold increase in the K_d of tryptophan uptake has been reported in rats which were presumably hyperammonemic and in acute hepatic failure following both portacaval anastomosis and hepatic artery ligation (Mans et al., 1979). The increase in K_d in the present studies was not apparently due to a direct effect of ammonia, since MSO-treated rats had K_d values similar to the controls (Table 4). It should be noted that the increased $AUI_{sucrose}$ observed in

both groups of ammonia-infused rats is not related to the observed rise in transport K_d . Regardless of MSO pretreatment, the BUI_{sucrose} was significantly higher after ammonia infusion, in contrast to the effect of MSO on transport K_d 's. The higher K_d may, therefore, be related to the increase in the brain content of glutamine and the LNAA which occurs after ammonia infusion (Jonung et al., 1984).

It is possible that the rise in BUI_{sucrose} represents a small amount of passage of this normally impermeant molecule (M.W. = 342) through openings in the blood-brain barrier which were not large enough to admit molecules of HRP (M.W. = 40,000). An alternative explanation, supported by the microscopy studies (Fig. 3B-3C), is that ammonia infusion resulted in a slower perfusion rate through a small population of cortical capillaries such that washout of sucrose from those capillaries was not as complete by 15 s after carotid injection as was the washout in control animals. Laurson and Diemer (1977) measured the diameters of capillaries in the cerebral cortex of rats after portacaval anastomosis to determine whether astrocyte swelling might reduce the capillary diameter; no reduction was observed. Astrocyte swelling may have been more severe in the present study since the degree of hyperammonemia was presumably greater than after portacaval anastomosis. Reduced flow through some capillaries may not have affected the overall rate of blood flow in cortex, which was not apparently reduced in the ammonia-infused rats under pentobarbital anesthesia (Table 3). Nonetheless, failure to correct for this change in BUI_{sucrose} would have resulted in higher values of K_d in the ammonia-infused rats than those which were calculated (Table 4).

Although the K_m of phenylalanine transport was not significantly affected by any of the treatments, this was not the case for the K_m of leucine transport. The K_m of leucine uptake was significantly increased by ammonia infusion, whereas pretreatment with MSO blocked this increase. Cardelli-Cangiano et al. (1984) studied the effect of ammonia on the uptake of amino acids by isolated brain microvessels. In that study the V_{max} , but not K_m , of leucine uptake was increased in the presence of 0.25 mM ammonium bicarbonate, whereas both the V_{max} and K_m of leucine uptake were increased in the presence of 1.0 mM ammonium bicarbonate. In the present studies, the circulating ammonia concentrations were presumably about 1.0 mM (Jonung et al., 1984). Mans et al. (1982) presented evidence that, whereas portacaval anastomosis resulted in increased clearance by brain of circulating phenylalanine and leucine, the increase in phenylalanine clearance was much greater than the increase in leucine clearance. Although the magnitude and direction of changes, if any, in kinetic parameters of amino acid transport cannot be inferred from the data of Mans et al. (1982), their data are consistent with a differential effect of portacaval anastomosis on one or more of the kinetic parameters of leucine and phenylalanine uptake. The present results, which suggest that hyperammonemia increases the K_m of leucine transport and the K_d of phenylalanine transport measured *in vivo*, are consistent with the results of Mans et al. (1982), although they do not explain the mechanism of such a differential effect.

The mechanism of blood-brain LNAA transport is not known. Sawada (1977) proposed that the gamma-glutamyl cycle, as originally put forward by Hailer (1973, 1974), could be modified to provide an enzymatic basis for

blood-brain LNAA transport. This modification suggested that γ -glutamyl-glutamine might serve as γ -glutamyl donor (in place of glutathione); γ -glutamyl transpeptidase, an enzyme which is present in the capillary endothelial cell (Orlowski et al., 1974), could regenerate γ -glutamyl-glutamine from the abundant brain pool of glutamine. In subsequent studies (Samuels and Fish, 1978; Samuels et al., 1978), evidence was presented that MSO, which inhibits both glutamine synthetase and γ -glutamylcysteine synthetase, an enzyme in the γ -glutamyl cycle, reduces the uptake of LNAA into brain in vivo. Recent studies (Samuels et al., 1983) have been presented as further support for glutamine exchange in blood-brain LNAA transport. If the mechanism of blood-brain LNAA transport involves, in whole or in part, the γ -glutamyl cycle, then the effect of MSO in the present studies may involve not only reduced glutamine synthesis, but also inhibition of γ -glutamylcysteine synthetase. Conversely, the stimulatory effect of ammonia may involve not only enhanced glutamine synthesis but also potential, unknown effects of ammonia on steps in the γ -glutamyl cycle.

On the other hand, if blood-brain transport of the LNAA is mediated by a "mobile carrier" mechanism, high concentrations of glutamine and other LNAA in brain extracellular fluid or within capillary endothelial cells may increase the rate of carrier return to the plasma side of the blood-brain barrier. Such a phenomenon, termed transacceleration or accelerative exchange diffusion, may be interpreted as enhanced mobility of an occupied carrier compared to an unoccupied one. The increase in V_{max} in ammonia-infused rats may be thought of, then, as increased availability of carriers

at the luminal surface. MSO may prevent the rise in $V_{\max}$ by preventing the rise in brain of glutamine and other LNAA and thus preventing accelerated exchange.

The present studies clearly show that hyperammonemia does not result in changes in kinetic parameters of LNAA transport across the blood-brain barrier if the animals have been pretreated with MSO. Taken together with the previously reported observation that MSO reduces brain LNAA concentrations in ammonia-infused (Jonung et al., 1984) or portacaval-shunted rats (Rigotti et al., 1984), these studies strongly suggest that hyperammonemia influences brain LNAA concentrations and blood-brain transport via synthesis in brain of glutamine. These studies, therefore, support the so-called "unified hypothesis" of James et al. (1979), which attempted to link the changes in brain amino acid and neurotransmitter metabolism seen in hepatic encephalopathy with the hyperammonemia of that condition. Since the effect of MSO is not limited to inhibition of glutamine synthetase, and since the mechanism of blood-brain LNAA transport is not known, the present results do not unequivocally assign glutamine a role in blood-brain LNAA transport in hyperammonemia. The present results also leave unresolved the question of whether the rise in brain LNAA levels precedes or follows the changes in blood-brain transport. The ammonia-infused rat appears to be a reproducible and relatively simple model in which to study the relationship of these phenomena.

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TABLE 1

Effect of Treatments on Brain Ammonia and Brain Glutamine

	Control	NH ₃	MSO, NH ₃
Brain ammonia (μmol/g)	0.20 ± 0.02 (6)	1.6 ± 0.2 (6) ^a	2.8 ± 0.2 (6) ^a
Brain glutamine (μmol/g)	5.5 ± 0.5 (6)	22.9 ± 0.5 (87) ^a	4.4 ± 0.3 (78)

Data expressed as mean ± SEM for number of rats given in parentheses.

^a Differs significantly from control value, $p < 0.01$ at least.

TABLE 2
Values of Cerebral Blood Flow and E_{HOH}

Treatment:	BUI_{HOH}^a (%)	K_B^b (min^{-1})	E_{HOH}^c (%)	F ($\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$)	BUI_{sucrose}^d (%)
None	89 ± 2	0.65 ± 0.07	80 ± 2	0.56 ± 0.06	1.7 ± 0.2 (11)
NH_4^+ salts	88 ± 6	0.81 ± 0.05	76 ± 5	0.71 ± 0.08	3.9 ± 0.7 (8)
MSO, NH_4^+ salts	87 ± 6	0.65 ± 0.08	78 ± 5	0.56 ± 0.01	3.4 ± 0.2 (5)

^a BUI_{HOH} for $^3\text{[H]}$ -water at 15 sec after carotid injection relative to $^{14}\text{[C]}$ -butanol. Data are mean $\pm$ S.E.M. (n = four to six animals).

^b Rate constants $\pm$ S.E.M. of $^{14}\text{[C]}$ -butanol efflux from brain as calculated from a linear regression of butanol washout data.

^c $E_{HOH} = (BUI_{HOH}) e^{-K_B t}$; t = 10 sec, as described by Pardridge et al. (1982).

^d Values (mean $\pm$ S.E.M.) of ammonia-infused groups differ significantly from control group; $p < 0.05$ at least.

TABLE 3
BUI-Phenylalanine and BUI-Leucine at Different Concentrations
in Injection Solution

Inj sol (nmol/ml)	BUI-Phenylalanine		
	Control	NH ₃	MSO, NH ₃
4	56.6 $\pm$ 2.5 (6)	72.5 $\pm$ 4.2 (6)	53.9 $\pm$ 2.1 (4)
37	48.6 $\pm$ 1.6 (6)	70.2 $\pm$ 1.0 (3)	53.2 $\pm$ 2.0 (3)
68	41.3 $\pm$ 2.6 (4)	56.6 $\pm$ 1.9 (4)	34.1 $\pm$ 2.8 (3)
129	24.8 $\pm$ 1.3 (5)	46.0 $\pm$ 2.2 (4)	28.6 $\pm$ 2.1 (3)
254	20.8 $\pm$ 2.2 (4)	37.2 $\pm$ 1.7 (4)	21.0 $\pm$ 0.3 (3)
504	20.3 $\pm$ 1.5 (4)	32.4 $\pm$ 0.5 (3)	18.6 $\pm$ 2.7 (4)
1004	14.1 $\pm$ 1.1 (4)	19.4 $\pm$ 0.7 (3)	13.1 $\pm$ 2.4 (4)
2004	8.6 $\pm$ 0.4 (4)	19.2 $\pm$ 1.6 (4)	9.3 $\pm$ 0.6 (4)
4004	7.6 $\pm$ 1.2 (5)	20.0 $\pm$ 1.3 (6)	7.4 $\pm$ 2.0 (4)
8004	7.3 $\pm$ 0.9 (4)	17.6 $\pm$ 1.0 (6)	9.4 $\pm$ 0.7 (4)

Inj sol (nmol/ml)	BUI-Leucine		
	Control	NH ₃	MSO, NH ₃
8	40.9 $\pm$ 1.5 (6)	52.9 $\pm$ 1.6 (4)	44.4 $\pm$ 2.8 (5)
40	34.5 $\pm$ 1.8 (5)	48.8 $\pm$ 2.1 (4)	37.2 $\pm$ 4.7 (3)
71	34.1 $\pm$ 1.8 (4)	48.8 $\pm$ 3.9 (6)	30.8 $\pm$ 4.8 (3)
133	22.6 $\pm$ 0.8 (3)	46.7 $\pm$ 1.8 (3)	25.0 $\pm$ 3.0 (3)
258	20.5 $\pm$ 0.4 (3)	36.1 $\pm$ 1.3 (4)	15.0 $\pm$ 2.1 (5)
508	15.0 $\pm$ 0.1 (3)	23.5 $\pm$ 1.7 (3)	11.0 $\pm$ 0.9 (5)
1008	11.8 $\pm$ 0.8 (4)	21.5 $\pm$ 0.9 (3)	8.4 $\pm$ 1.1 (3)
2008	9.4 $\pm$ 0.6 (3)	14.9 $\pm$ 0.2 (3)	4.1 $\pm$ 0.5 (5)
4008	3.7 $\pm$ 0.8 (7)	6.5 $\pm$ 1.1 (7)	2.1 $\pm$ 0.4 (5)
8008	3.3 $\pm$ 0.4 (8)	5.2 $\pm$ 0.8 (7)	1.7 $\pm$ 0.5 (5)

Values are given as mean $\pm$ S.E.M. The number of rats is given in parentheses.

The BUI_{sucrose} has been subtracted from each value.

TABLE 4

Effects of Ammonia and MSO on the Kinetics of Blood-Brain Transport
of Phenylalanine and Leucine into Brain Cortex

Phenylalanine			
Treatment:	K_m^a (mM)	V_{max}^a (nmol·min ⁻¹ ·g ⁻¹)	K_d^a (ml·min ⁻¹ ·g ⁻¹)
None	0.09 ± 0.01	21 ± 3	0.033 ± 0.005
NH ₄ ⁺ salts	0.12 ± 0.02	38 ± 6 ^{b, c}	0.090 ± 0.007 ^{b, c}
MSO, NH ₄ ⁺ salts	0.10 ± 0.02	21 ± 3	0.031 ± 0.006
Leucine			
None	0.20 ± 0.03	39 ± 4	0.013 ± 0.003
NH ₄ ⁺ salts	0.40 ± 0.08 ^{b, c}	108 ± 23 ^{b, c}	0.021 ± 0.012
MSO, NH ₄ ⁺ salts	0.11 ± 0.02	20 ± 3	0.008 ± 0.005

^a Estimates ± S.D. are calculated from non-linear regression analysis of data.

^b Differs significantly from same parameter in untreated control rats;
p<0.05 at least.

^c Differs significantly from same parameter in MSO-pretreated rats;
p<0.05 at least.

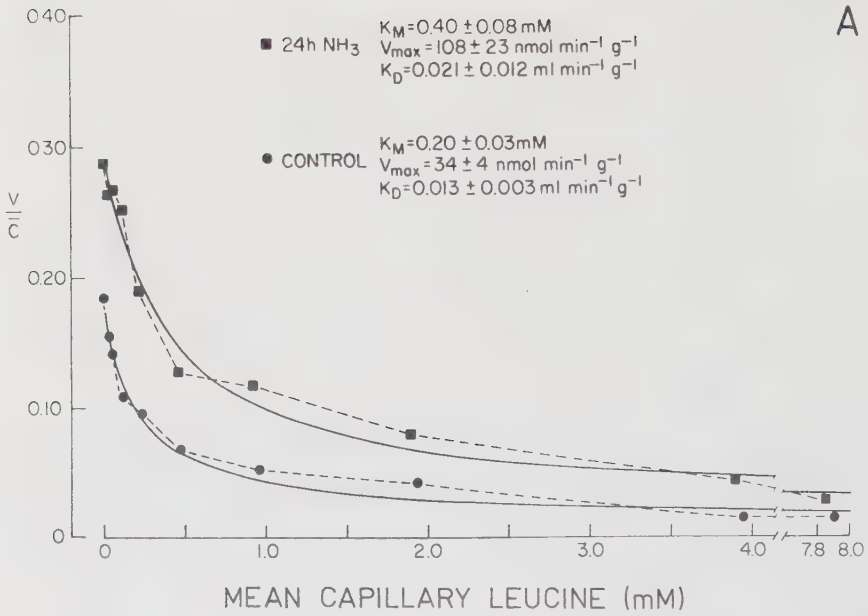
Fig. 1 The unidirectional clearance rate, V/C ($\text{ml} \cdot \text{min}^{-1} \cdot \text{g}^{-1}$), plotted as a function of mean capillary leucine (A) or phenylalanine (B) in control rats (●) and ammonia-infused rats without MSO (■). The points connected by the broken lines represent the mean clearance and capillary amino acid concentration for the number of rats shown in Table 3. Individual, rather than mean, values were fitted to $V/\bar{C} = [V_{\text{max}}/(K_m + \bar{C})] + K_d$ using least squares nonlinear regression. The line of best fit is the solid curve. Data from the MSO-treated rats are not shown, but would be close to the values shown for control rats.

Fig. 2 Electron micrographs from brain of rats receiving ammonium salt infusion without MSO.

- (A) Capillary in cerebral cortex showing nucleus (crescent-shaped thickening), open lumen (L) and swelling of astrocyte end-foot (ef). No HRP reaction product was seen outside capillaries. Scale bar = 2 microns.
- (B) Capillary in cerebellum also showing extensive perivascular swelling. Scale bar = 3 microns.
- (C) High magnification of small segment of capillary wall showing rare pinocytotic vesicle (V) containing HRP reaction product, intact tight junction (marked by arrows) and basement membrane (upper arrow). Scale bar = 0.2 microns.
- (D) High magnification of segment of capillary wall from cerebral cortex showing red blood cell in lumen (upper right), intact tight junction (arrows), basement membrane, and swollen astrocyte end-feet (ef). Scale bar = 0.2 microns.

- Fig. 3** (A) Capillary in cortex of MSO-treated rat, showing swollen astrocyte end-feet (ef). The membranes between adjacent astrocyte processes appear to be intact. Scale bar = 2 microns.
- (B) MSO-treated rat. Extensive perivascular swelling nearly surrounding the capillary and apparently compressing it. An erythrocyte (C) may be trapped inside the lumen. Scale bar = 2 microns.
- (C) Capillary in cortex of ammonia-infused rat not treated with MSO. The capillary is surrounded by extensive swelling and the lumen (L) appears to have partially collapsed. Scale bar = 2 microns.

A



B

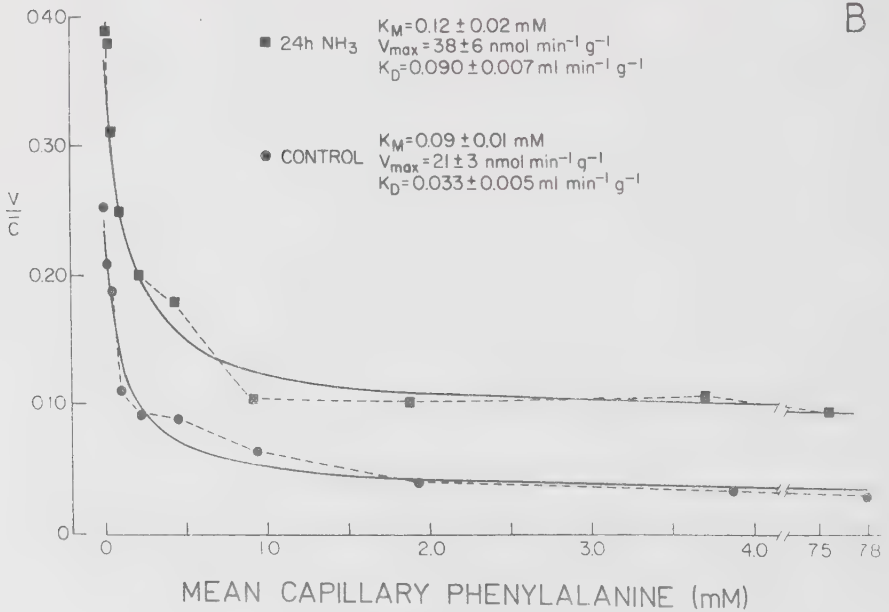


FIGURE 1

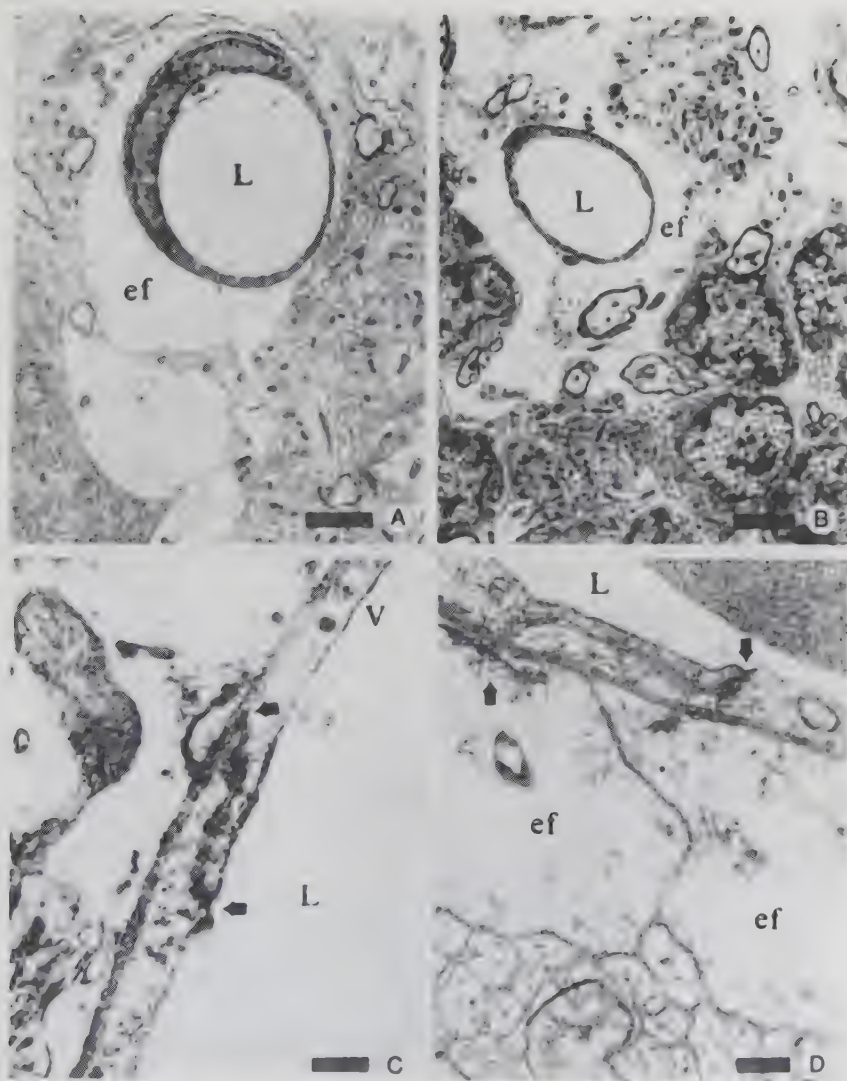


FIGURE 2

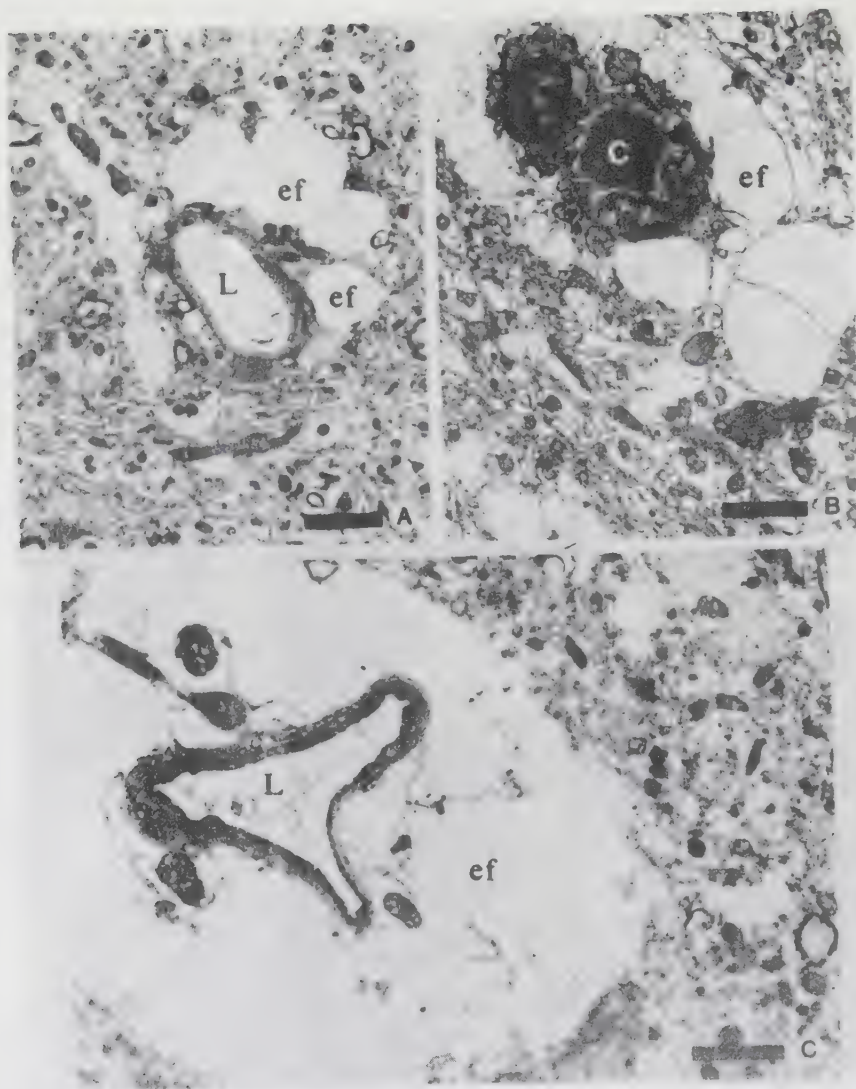


FIGURE 3

Indole Amines and Amino Acids in Various Brain Regions after Infusion of Branched Chain Amino Acids into Hepatectomized Rats

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Key Words. Amino acids · Indole amines · Hepatectomy · Brain regions

Abstract. This study was designed to determine regional changes of amino acids and indole amines in the brain and possible interactions between amino acids and indole amines 18 h after hepatectomy in rats. Hepatectomy and glucose infusion alone resulted in a profound increase of most large neutral amino acids (LNAA) in plasma and in the brain except for the branched-chain amino acids (BCAA), which maintained normal or somewhat lower values in plasma. Hepatectomy and infusion of glucose combined with BCAA sharply reduced the plasma and brain amino acid concentrations of other LNAA. Simultaneously the concentrations of serotonin and 5-hydroxyindoleacetic acid were decreased in all brain regions. In both groups of hepatectomized rats there were regional variations of the amino acid and the indole amine concentrations in the brain, but the response to BCAA infusion was generally the same in all brain regions. No difference in survival between the 2 groups could be found.

Introduction

Accumulation in the brain of large neutral amino acids (LNAA) such as tyrosine, phenylalanine and tryptophan may disturb the neurotransmitter metabolism and thereby contribute to hepatic coma and death [6]. High brain levels of tryptophan stimulate serotonin synthesis in patients dying in hepatic coma [16] and in rats after hepatectomy [14] or portocaval anastomosis [2, 4, 5].

High plasma concentrations of the branched-chain amino acids (BCAA) have been proposed to reduce the accumulation of other LNAA in the brain by means of increased competition for transport across the blood-brain barrier, and by a reduction of other LNAA in plasma as a result of a direct stimulation of the protein metabolism in the muscles by the BCAA [7, 8]. Several conflicting reports of the effect of BCAA treatment on hepatic encephalopathy in humans have

been presented and this subject is still controversial [3, 8, 20, 21].

This study of regional brain amino acids, serotonin and 5-hydroxyindoleacetic acid in hepatectomized rats was undertaken with particular attention to the following objectives: to determine whether changes in indole amine concentrations were more profound in some regions than in others, to consider possible correlations between regional amino acid levels and regional indole amine metabolism, and to determine the effects on regional amino acids and indole amines of reducing the influx of LNAA into brain by infusion of BCAA.

Hepatectomy was chosen as an experimental model of acute hepatic failure to ensure that lysis of necrotic liver tissue did not contribute to the high plasma levels of amino acids.

Material and Methods

Male Sprague-Dawley rats (initial weight 225–275 g) underwent hepatectomy in three stages under ether anesthesia as follows: (a) division of inferior vena cava above the renal veins; (b) 4 weeks later, an end-to-side portocaval anastomosis without ligation of the pancreaticoduodenal vein and, (c) 1 week later, removal of all liver parenchyma after ligation of the hepatic artery, biliary duct and the inferior vena cava [15]. Control groups underwent either steps (a) and (b) and a sham operation at step (c) (group C) or step (a) and sham operations at steps (b) and (c) (group D). After the third operation, a silastic catheter (Dow Corning; 0.76 mm inner diameter $\times$ 1.65 mm outer diameter) was placed in the jugular vein for infusion of solutions. Food was not available during the infusion. Hepatectomized animals were infused either with a solution containing 10% glucose, 143 mM NaCl, 13 mM K_2HPO_4 , 2.3 mM $MgSO_4$, and 1.5 mM $ZnCl_2$, pH 7.4 (group A) or the same solution plus 0.24 M BCAA (0.08 M each of valine, leucine and isoleucine) (group B). The control groups C and D received the glucose solution. The solu-

tions were infused at a rate of 0.6 ml/h/100 g body weight. Rats were housed in individual cages and warmed by proximity to a light bulb. Rectal temperature was maintained at 35–37 °C.

The rats were decapitated at 18 h after hepatectomy or sham operation. Blood from the neck was collected into heparinized beakers and plasma was separated by centrifugation and kept frozen at –72 °C until it was analyzed. The brain was removed and dissected into the following regions: hypothalamus, cortex, striatum, hippocampus, diencephalon, mesencephalon, cerebellum and pons medulla. The brain was dissected regionally according to Glowinski and Iversen [11] with the following exceptions: (1) the mesencephalon was separated from the remaining part of the diencephalon and the septal area by a vertical cut just anterior to the superior colliculus, and (2) the hypothalamic area was dissected to a depth of approximately 2.5 mm from the optic chiasm to the posterior mamillary area, bound laterally by the choroid fissure. The dissection was carried out on a plate chilled on ice and the samples were quickly frozen in liquid nitrogen and kept frozen at –72 °C until analyzed.

Two experiments with hepatectomized and control rats were prepared. In experiment (I), tryptophan, serotonin, and 5-hydroxyindoleacetic acid (5-HIAA) were determined in eight brain regions, and in experiment (II), amino acid concentrations were measured in plasma and in the eight regions. The following amino acids were studied: threonine (THR), serine (SER), glutamic acid (GLU), glutamine (GLN), glycine (GLY), alanine (ALA), valine (VAL), methionine (MET), isoleucine (ILE), leucine (LEU), tyrosine (TYR), phenylalanine (PHE), tryptophan (TRP), lysine (LYS), histidine (HIS) and arginine (ARG).

Amino acids were determined by using a Beckman 121-MB amino acid analyzer with automatic integration. Plasma was deproteinized by mixing 0.5 ml plasma with 1.5 ml 5% sulfosalicylic acid (SSA) (pH 1.7), containing 133 nmol/ml thienylalanine as internal standard. After vortexing, the samples were centrifuged (20 min, 30,000 g at 4 °C) and passed through a 0.45- μ m Millipore filter.

The brain pieces were homogenized in 3 times their weight of 5% SSA (pH 1.4) containing 133 nmol/ml thienylalanine. Each brain sample was analyzed twice, once at the 1:4 dilution of the homogenate for the low-concentration amino acids and again after further 1:25 dilution to measure the high concentration amino acids, glutamate and glutamine.

TRP, serotonin and 5-HIAA in the brain regions were assayed by reverse-phase liquid chromatography using an Altex Ultrasphere ODS (C-18), 5- μ m particle size (25 $\times$ 0.45 cm), protected by a small guard column (4.5 $\times$ 0.32 cm), and packed with 10- μ m Ultrasphere ODS. The brain regions were weighed and homogenized in (10 $\times$ weight) 1 *N* formic/acetone (v/v: 10/1) without internal standard in order to maintain constant volumes and to keep the concentrations of salt and H₂O proportional in all samples. After centrifugation (1,000 g, 10 min, 4 °C), the samples were divided by transferring 2.5 ml of supernatant to each of two tubes containing 7.5 ml of heptane/chloroform. The tubes were shaken for 10 min and centrifuged (2,500 g, 10 min, 4 °C) and the organic layer and lipid interface were discarded. Activated alumina (100 mg) was added to the aqueous phase and the pH of the mixture was adjusted to 8.2–8.4 with 0.5 *M* Tris buffer containing 2 *mM* EDTA and 0.03% sodium metabisulfate. The alumina was washed twice with 10 ml distilled deionized water. The aqueous phase of the duplicate sample was transferred to a small glass tube and reduced to dryness under a nitrogen gas stream. The dried sample was redissolved in 0.8 ml of mobile phase and the sample was centrifuged to remove any particulate matter before analysis.

Statistics

Multivariate analysis of variance was performed on amines and amino acids to determine differences among the treatment groups. Subsequently Duncan's test was used for comparisons between treatment groups.

Results

About 60% of the rats survived 18 h after hepatectomy. No difference in survival or frequency of coma could be observed between rats infused with BCAA or glucose. In this paper rats infused with glucose after hepatectomy are referred to as group A, rats infused with BCAA after hepatectomy as group B, rats infused with glucose after portocaval anastomosis as group C, and rats in-

fused with glucose after sham operation as group D. All differences were significant ($p < 0.05$ at least), unless otherwise noted.

Plasma Amino Acids (tables I and II)

In group A the concentrations of all amino acids were higher than in groups C and D, except for ILE, VAL, LEU and GLU, which were unchanged. In group B, compared to group A, the concentrations of PHE, TYR, MET, LYS, THR, TRP and HIS were lower while the concentrations of GLU, GLN, GLY, SER, ALA and the BCAA were higher.

Brain Amino Acids (tables I and II)

Group C vs. Group D. GLN, TYR and PHE were higher in all regions in group C. HIS, MET, THR, LYS and GLY were higher in all regions in group C, although not significantly in all regions. The concentrations of GLU in hypothalamus were higher in group C than in group D. SER was higher in the pons medulla and lower in the striatum in group C than in group D.

Group A vs. Group C. GLN, ALA, GLY, HIS, TYR, PHE, MET, THR and LYS were higher in all regions in group A than in group C. However, these changes were not significant in all regions for ALA, MET and GLY. SER was lower in the striatum and higher in the pons medulla in group A than in group C. GLU was lower in all regions of group A except for the cerebellum.

Group B vs. Group A. The BCAA were higher in all regions of group B compared to group A. GLN, GLU, ALA and GLY were significantly higher in a few regions in group B. In group B, PHE, MET, TYR, THR, HIS and LYS were lower in all regions. The concentration of SER was increased in the mesencephalon in group B.

Table I. Regional amino acid concentrations ($\mu\text{mol/g}$ wet weight; means $\pm$ SEM) after hepatectomy and in control rats

	Glu	Gln	Gly	Ala	Leu	Tyr	Phe
Hypothalamus							
Hep-gluc	6.0 $\pm$ 0.56 ^b	24.7 $\pm$ 1.27 ^a	1.39 $\pm$ 0.06 ^a	0.88 $\pm$ 0.27 ^b	0.06 $\pm$ 0.01 ^b	0.49 $\pm$ 0.01 ^a	0.45 $\pm$ 0.02 ^a
Hep-BCAA	5.8 $\pm$ 0.35 ^b	24.8 $\pm$ 1.40 ^a	1.45 $\pm$ 0.04 ^a	1.53 $\pm$ 0.33 ^a	0.41 $\pm$ 0.05 ^a	0.09 $\pm$ 0.01 ^c	0.06 $\pm$ 0.01 ^c
PC-shunt	9.4 $\pm$ 0.55 ^a	12.9 $\pm$ 1.14 ^b	1.30 $\pm$ 0.1 ^a	0.50 $\pm$ 0.05 ^b	0.09 $\pm$ 0.01 ^b	0.27 $\pm$ 0.03 ^b	0.19 $\pm$ 0.02 ^b
Sham-op	7.1 $\pm$ 0.42 ^b	5.8 $\pm$ 0.45 ^c	1.10 $\pm$ 0.05 ^b	0.42 $\pm$ 0.03 ^b	0.07 $\pm$ 0.05 ^b	0.09 $\pm$ 0.01 ^c	0.08 $\pm$ < 0.01 ^c
Cortex							
Hep-gluc	8.0 $\pm$ 0.52 ^b	21.1 $\pm$ 1.17 ^b	1.10 $\pm$ 0.03 ^a	1.71 $\pm$ 0.51 ^a	0.08 $\pm$ 0.01 ^b	0.62 $\pm$ 0.03 ^a	0.58 $\pm$ 0.02 ^a
Hep-BCAA	8.9 $\pm$ 0.45 ^b	24.1 $\pm$ 1.30 ^a	1.16 $\pm$ 0.03 ^a	2.41 $\pm$ 0.42 ^a	0.41 $\pm$ 0.04 ^a	0.11 $\pm$ 0.02 ^c	0.07 $\pm$ 0.01 ^c
PC-shunt	11.2 $\pm$ 0.23 ^a	10.0 $\pm$ 0.90 ^c	0.77 $\pm$ 0.03 ^b	0.57 $\pm$ 0.02 ^b	0.06 $\pm$ < 0.01 ^b	0.23 $\pm$ 0.02 ^b	0.15 $\pm$ 0.01 ^b
Sham-op	10.6 $\pm$ 0.36 ^a	5.3 $\pm$ 0.45 ^d	0.64 $\pm$ 0.03 ^c	0.57 $\pm$ 0.02 ^b	0.05 $\pm$ < 0.01 ^b	0.08 $\pm$ 0.01 ^c	0.06 $\pm$ 0.07 ^c
Striatum							
Hep-gluc	5.4 $\pm$ 0.04 ^c	16.8 $\pm$ 1.25 ^b	0.66 $\pm$ 0.03 ^b	1.79 $\pm$ 0.53 ^b	0.07 $\pm$ 0.08 ^b	0.50 $\pm$ 0.03 ^a	0.44 $\pm$ 0.02 ^a
Hep-BCAA	6.8 $\pm$ 0.35 ^b	21.5 $\pm$ 0.94 ^a	0.78 $\pm$ 0.03 ^a	3.12 $\pm$ 0.44 ^a	0.38 $\pm$ 0.04 ^a	0.09 $\pm$ 0.01 ^c	0.07 $\pm$ 0.01 ^c
PC-shunt	10.7 $\pm$ 0.31 ^a	11.6 $\pm$ 0.96 ^c	0.64 $\pm$ 0.02 ^b	0.65 $\pm$ 0.23 ^c	0.08 $\pm$ < 0.01 ^b	0.24 $\pm$ 0.02 ^b	0.17 $\pm$ 0.01 ^b
Sham-op	11.3 $\pm$ 0.15 ^a	7.0 $\pm$ 0.60 ^d	0.57 $\pm$ 0.01 ^c	0.62 $\pm$ 0.02 ^c	0.07 $\pm$ < 0.01 ^b	0.10 $\pm$ 0.01 ^c	0.09 $\pm$ 0.01 ^c
Hippocampus							
Hep-gluc	7.8 $\pm$ 0.59 ^c	22.3 $\pm$ 1.20 ^b	1.03 $\pm$ 0.05 ^a	1.89 $\pm$ 0.48 ^a	0.08 $\pm$ 0.01 ^b	0.55 $\pm$ 0.03 ^a	0.52 $\pm$ 0.02 ^a
Hep-BCAA	9.1 $\pm$ 0.45 ^b	26.0 $\pm$ 1.04 ^a	1.14 $\pm$ 0.04 ^a	2.73 $\pm$ 0.47 ^a	0.39 $\pm$ 0.04 ^a	0.10 $\pm$ 0.01 ^c	0.08 $\pm$ 0.01 ^c
PC-shunt	11.4 $\pm$ 0.29 ^a	9.7 $\pm$ 1.11 ^c	0.77 $\pm$ 0.03 ^b	0.69 $\pm$ 0.03 ^b	0.06 $\pm$ < 0.01 ^b	0.22 $\pm$ 0.01 ^b	0.18 $\pm$ 0.17 ^b
Sham-op	11.4 $\pm$ 0.32 ^a	4.7 $\pm$ 0.33 ^d	0.67 $\pm$ 0.03 ^b	0.69 $\pm$ 0.03 ^b	0.06 $\pm$ < 0.01 ^b	0.09 $\pm$ 0.02 ^c	0.08 $\pm$ 0.02 ^c
Diencephalon							
Hep-gluc	6.8 $\pm$ 0.54 ^b	23.4 $\pm$ 1.76 ^a	0.98 $\pm$ 0.04 ^a	1.15 $\pm$ 0.31 ^a	0.07 $\pm$ 0.01 ^b	0.49 $\pm$ 0.02 ^a	0.44 $\pm$ 0.01 ^a
Hep-BCAA	7.5 $\pm$ 0.32 ^b	25.0 $\pm$ 1.24 ^a	0.98 $\pm$ 0.04 ^a	1.48 $\pm$ 0.29 ^a	0.35 $\pm$ 0.04 ^a	0.09 $\pm$ 0.01 ^c	0.07 $\pm$ 0.01 ^c
PC-shunt	9.5 $\pm$ 0.31 ^a	10.5 $\pm$ 1.07 ^b	0.82 $\pm$ 0.04 ^b	0.39 $\pm$ 0.04 ^b	0.06 $\pm$ < 0.01 ^b	0.21 $\pm$ 0.02 ^b	0.19 $\pm$ 0.03 ^b
Sham-op	9.2 $\pm$ 0.30 ^a	4.8 $\pm$ 0.33 ^c	0.80 $\pm$ 0.03 ^b	0.40 $\pm$ 0.01 ^b	0.05 $\pm$ < 0.01 ^b	0.08 $\pm$ 0.01 ^c	0.07 $\pm$ 0.01 ^c
Mesencephalon							
Hep-gluc	5.9 $\pm$ 0.60 ^b	29.1 $\pm$ 2.22 ^a	1.53 $\pm$ 0.10 ^a	0.95 $\pm$ 0.28 ^a	0.08 $\pm$ 0.01 ^b	0.46 $\pm$ 0.03 ^a	0.42 $\pm$ 0.01 ^a
Hep-BCAA	6.6 $\pm$ 0.58 ^b	30.6 $\pm$ 1.29 ^a	1.66 $\pm$ 0.06 ^a	1.44 $\pm$ 0.30 ^a	0.48 $\pm$ 0.07 ^a	0.09 $\pm$ 0.15 ^c	0.06 $\pm$ 0.01 ^c
PC-shunt	9.3 $\pm$ 0.23 ^a	11.3 $\pm$ 1.32 ^b	1.17 $\pm$ 0.02 ^b	0.31 $\pm$ 0.01 ^b	0.06 $\pm$ < 0.01 ^b	0.16 $\pm$ 0.02 ^b	0.11 $\pm$ 0.01 ^b
Sham-op	9.5 $\pm$ 0.13 ^a	5.0 $\pm$ 0.40 ^c	1.13 $\pm$ 0.02 ^b	0.30 $\pm$ < 0.01 ^b	0.06 $\pm$ < 0.01 ^b	0.07 $\pm$ 0.01 ^c	0.06 $\pm$ 0.01 ^c
Cerebellum							
Hep-gluc	10.4 $\pm$ 0.77 ^a	36.1 $\pm$ 2.59 ^a	0.59 $\pm$ 0.02 ^b	1.14 $\pm$ 0.28 ^b	0.08 $\pm$ 0.02 ^b	0.64 $\pm$ 0.04 ^a	0.52 $\pm$ 0.02 ^a
Hep-BCAA	10.8 $\pm$ 0.87 ^a	34.3 $\pm$ 1.45 ^a	0.65 $\pm$ 0.01 ^a	1.77 $\pm$ 0.28 ^a	0.35 $\pm$ 0.05 ^a	0.10 $\pm$ 0.01 ^c	0.06 $\pm$ 0.01 ^c
PC-shunt	10.1 $\pm$ 0.24 ^a	11.9 $\pm$ 1.14 ^b	0.57 $\pm$ 0.02 ^b	0.56 $\pm$ 0.01 ^c	0.04 $\pm$ < 0.01 ^b	0.24 $\pm$ 0.03 ^b	0.15 $\pm$ 0.02 ^b
Sham-op	10.2 $\pm$ 0.25 ^a	6.1 $\pm$ 0.51 ^c	0.56 $\pm$ 0.02 ^b	0.59 $\pm$ 0.02 ^c	0.04 $\pm$ < 0.01 ^b	0.09 $\pm$ 0.01 ^c	0.08 $\pm$ 0.01 ^c
Pons medulla							
Hep-gluc	5.1 $\pm$ 0.39 ^b	21.1 $\pm$ 1.52 ^a	3.10 $\pm$ 0.10 ^a	0.87 $\pm$ 0.17 ^a	0.06 $\pm$ 0.01 ^b	0.45 $\pm$ 0.02 ^a	0.45 $\pm$ 0.01 ^a
Hep-BCAA	5.8 $\pm$ 0.27 ^a	23.2 $\pm$ 1.29 ^a	3.29 $\pm$ 0.08 ^a	1.19 $\pm$ 0.22 ^a	0.34 $\pm$ 0.05 ^a	0.08 $\pm$ 0.01 ^c	0.06 $\pm$ 0.01 ^c
PC-shunt	6.3 $\pm$ 0.10 ^a	7.3 $\pm$ 0.79 ^b	2.97 $\pm$ 0.08 ^b	0.42 $\pm$ 0.02 ^b	0.05 $\pm$ < 0.01 ^b	0.17 $\pm$ 0.02 ^b	0.13 $\pm$ 0.01 ^b
Sham-op	6.1 $\pm$ 0.08 ^a	3.4 $\pm$ 0.28 ^c	2.16 $\pm$ 0.05 ^c	0.31 $\pm$ < 0.01 ^b	0.05 $\pm$ < 0.01 ^b	0.07 $\pm$ < 0.01 ^c	0.06 $\pm$ 0.01 ^c
Plasma							
Hep-gluc	0.07 $\pm$ 0.01 ^b	2.53 $\pm$ 0.15 ^b	0.91 $\pm$ 0.05 ^b	0.84 $\pm$ 0.08 ^b	0.08 $\pm$ < 0.01 ^b	0.40 $\pm$ 0.03 ^a	0.40 $\pm$ 0.04 ^a
Hep-BCAA	0.14 $\pm$ 0.02 ^a	6.26 $\pm$ 0.33 ^a	1.10 $\pm$ 0.05 ^a	4.22 $\pm$ 0.27 ^a	1.28 $\pm$ 0.22 ^a	0.21 $\pm$ 0.01 ^b	0.14 $\pm$ 0.01 ^b
PC-shunt	0.05 $\pm$ < 0.01 ^b	0.86 $\pm$ 0.04 ^c	0.31 $\pm$ 0.02 ^c	0.36 $\pm$ 0.04 ^c	0.07 $\pm$ 0.01 ^c	0.11 $\pm$ 0.01 ^c	0.09 $\pm$ 0.01 ^c
Sham-op	0.06 $\pm$ 0.01 ^b	0.71 $\pm$ 0.03 ^c	0.29 $\pm$ 0.02 ^c	0.41 $\pm$ 0.05 ^c	0.08 $\pm$ 0.01 ^b	0.07 $\pm$ < 0.01 ^c	0.06 $\pm$ < 0.01 ^c

Hep-gluc: n = 8; Hep-BCAA: n = 10; PC-shunt: n = 10; Sham-op: n = 8.

Data with different letters are statistically significant ($p < 0.05$ at least) from each other, starting with the letter 'a' as the highest value or group of values. Comparison is only within one region and one component. For statistical methods, see 'Materials and Methods'.

Table II. Regional amino acid concentrations ($\mu\text{mol/g}$ wet weight, means $\pm$ SEM) after hepatectomy and in control rats

	Thr	Ser	Val	Met	Ile	Trp	Lys	His
Hypothalamus								
Hep-gluc	0.81 ± 0.05^a	0.73 ± 0.05^a	0.06 ± 0.01^b	0.15 ± 0.02^a	$0.06 \pm < 0.01^b$	—	0.82 ± 0.03^a	0.52 ± 0.04^a
Hep-BCAA	0.18 ± 0.02^d	0.69 ± 0.02^a	0.46 ± 0.06^a	$0.02 \pm < 0.01^d$	0.27 ± 0.04^a	—	$0.44 \pm 0.03^{b,c}$	$0.12 \pm 0.01^{b,c}$
PC-shunt	0.63 ± 0.05^b	0.73 ± 0.06^a	0.07 ± 0.01^b	0.11 ± 0.01^b	0.03 ± 0.01^b	—	0.50 ± 0.05^b	0.17 ± 0.01^b
Sham-op	0.46 ± 0.02^c	0.57 ± 0.04^a	0.05 ± 0.01^b	0.07 ± 0.01^c	$0.03 \pm < 0.01^b$	—	0.36 ± 0.03^c	0.07 ± 0.01^c
Cortex								
Hep-gluc	0.76 ± 0.05^a	0.93 ± 0.03^a	0.08 ± 0.01^b	0.19 ± 0.02^a	$0.02 \pm < 0.01^b$	—	0.51 ± 0.03^a	0.68 ± 0.05^a
Hep-BCAA	0.19 ± 0.02^d	0.94 ± 0.03^a	0.43 ± 0.04^a	0.03 ± 0.01^c	0.27 ± 0.03^a	—	0.31 ± 0.02^b	$0.14 \pm 0.02^{b,c}$
PC-shunt	0.57 ± 0.04^b	0.98 ± 0.03^b	$0.06 \pm < 0.01^b$	$0.09 \pm < 0.01^b$	$0.03 \pm < 0.01^b$	—	0.21 ± 0.01^c	0.15 ± 0.01^b
Sham-op	0.43 ± 0.02^c	0.93 ± 0.02^a	$0.06 \pm < 0.01^b$	$0.07 \pm < 0.01^b$	$0.02 \pm < 0.01^b$	—	0.16 ± 0.01^c	0.07 ± 0.01^b
Sinatum								
Hep-gluc	0.65 ± 0.06^a	0.83 ± 0.04^c	0.11 ± 0.01^b	0.15 ± 0.01^a	$0.04 \pm < 0.01^b$	—	0.46 ± 0.02^a	0.54 ± 0.04^a
Hep-BCAA	0.20 ± 0.02^b	$0.92 \pm 0.03^{b,c}$	0.36 ± 0.04^a	$0.03 \pm < 0.01^b$	0.24 ± 0.03^a	—	0.33 ± 0.02^b	$0.12 \pm 0.02^{b,c}$
PC-shunt	0.64 ± 0.04^b	0.99 ± 0.03^b	0.10 ± 0.01^b	0.14 ± 0.02^a	$0.05 \pm < 0.01^b$	—	0.34 ± 0.02^b	$0.15 \pm 0.02^{b,c}$
Sham-op	0.56 ± 0.02^a	1.10 ± 0.04^a	$0.09 \pm < 0.01^b$	$0.06 \pm < 0.01^b$	0.04 ± 0.01^b	—	0.21 ± 0.01^c	0.09 ± 0.01^b
Hippocampus								
Hep-gluc	0.70 ± 0.05^a	0.84 ± 0.04^a	0.08 ± 0.01^b	0.16 ± 0.02^a	$0.03 \pm < 0.01^b$	—	0.48 ± 0.02^a	0.62 ± 0.04^a
Hep-BCAA	0.17 ± 0.01^d	0.85 ± 0.03^a	0.41 ± 0.04^a	$0.02 \pm < 0.01^d$	0.27 ± 0.03^a	—	0.31 ± 0.02^b	$0.13 \pm 0.02^{b,c}$
PC-shunt	0.55 ± 0.04^b	0.85 ± 0.02^a	$0.06 \pm < 0.01^b$	0.09 ± 0.01^b	$0.03 \pm < 0.01^b$	—	0.23 ± 0.02^c	$0.15 \pm 0.02^{b,c}$
Sham-op	0.41 ± 0.01^c	0.81 ± 0.04^a	$0.06 \pm < 0.01^b$	$0.06 \pm < 0.01^c$	$0.03 \pm < 0.01^b$	—	0.17 ± 0.02^c	0.07 ± 0.01^c
Diencephalon								
Hep-gluc	0.79 ± 0.06^a	0.75 ± 0.03^a	0.07 ± 0.01^b	0.08 ± 0.02^a	$0.02 \pm < 0.01^b$	—	0.50 ± 0.03^a	0.54 ± 0.04^a
Hep-BCAA	0.19 ± 0.01^c	0.68 ± 0.02^a	0.35 ± 0.04^a	$0.01 \pm < 0.01^c$	0.22 ± 0.03^a	—	0.37 ± 0.03^b	$0.13 \pm 0.02^{b,c}$
PC-shunt	0.54 ± 0.05^b	0.63 ± 0.03^a	$0.05 \pm < 0.01^b$	$0.07 \pm < 0.01^{a,b}$	$0.02 \pm < 0.01^b$	—	$0.37 \pm < 0.01^b$	$0.15 \pm 0.01^{b,c}$
Sham-op	0.47 ± 0.02^c	0.60 ± 0.02^a	$0.06 \pm < 0.01^b$	$0.05 \pm < 0.01^b$	$0.02 \pm < 0.01^b$	—	0.24 ± 0.02^c	0.08 ± 0.01^c
Mesencephalon								
Hep-gluc	0.62 ± 0.06^a	0.41 ± 0.03^b	0.08 ± 0.01^b	0.15 ± 0.01^a	0.04 ± 0.01^b	—	0.53 ± 0.04^a	0.49 ± 0.04^a
Hep-BCAA	0.19 ± 0.02^d	0.49 ± 0.02^a	0.44 ± 0.07^a	$0.04 \pm < 0.01^d$	0.27 ± 0.05^a	—	0.36 ± 0.02^b	$0.13 \pm 0.02^{b,c}$
PC-shunt	0.51 ± 0.03^b	0.41 ± 0.01^b	$0.06 \pm < 0.01^b$	$0.09 \pm < 0.01^b$	$0.04 \pm < 0.01^b$	—	0.37 ± 0.02^b	$0.13 \pm 0.01^{b,c}$
Sham-op	0.41 ± 0.02^c	0.35 ± 0.01^b	$0.05 \pm < 0.01^b$	$0.07 \pm < 0.01^c$	$0.04 \pm < 0.01^b$	—	0.31 ± 0.02^c	0.06 ± 0.01^c
Cerebellum								
Hep-gluc	0.79 ± 0.05^a	0.61 ± 0.03^a	0.07 ± 0.01^b	0.20 ± 0.02^a	$0.04 \pm < 0.01^b$	—	0.67 ± 0.04^a	0.75 ± 0.05^a
Hep-BCAA	0.22 ± 0.01^d	0.64 ± 0.02^a	0.39 ± 0.06^a	$0.02 \pm < 0.01^d$	0.23 ± 0.04^a	—	0.48 ± 0.02^b	$0.14 \pm 0.02^{b,c}$
PC-shunt	0.68 ± 0.05^b	0.64 ± 0.02^a	$0.04 \pm < 0.01^b$	0.09 ± 0.01^b	$0.02 \pm < 0.01^b$	—	$0.47 \pm 0.02^{b,c}$	$0.20 \pm 0.02^{b,c}$
Sham-op	0.55 ± 0.02^c	0.68 ± 0.02^a	$0.05 \pm < 0.01^b$	$0.05 \pm < 0.01^c$	$0.02 \pm < 0.01^b$	—	0.40 ± 0.03^c	0.09 ± 0.01^c
Pons medulla								
Hep-gluc	0.79 ± 0.07^a	0.51 ± 0.02^a	0.07 ± 0.01^b	0.11 ± 0.01^a	$0.02 \pm < 0.01^b$	—	0.71 ± 0.04^a	0.51 ± 0.03^a
Hep-BCAA	0.20 ± 0.02^d	$0.54 \pm 0.02^{a,b}$	0.46 ± 0.06^a	$0.01 \pm < 0.01^c$	0.21 ± 0.03^a	—	0.46 ± 0.02^b	$0.13 \pm 0.02^{b,c}$
PC-shunt	0.63 ± 0.04^b	0.47 ± 0.01^b	$0.05 \pm < 0.01^b$	0.06 ± 0.01^b	$0.02 \pm < 0.01^b$	—	$0.45 \pm 0.02^{b,c}$	$0.12 \pm 0.01^{b,c}$
Sham-op	0.39 ± 0.02^c	0.34 ± 0.01^c	$0.04 \pm < 0.01^b$	$0.05 \pm < 0.01^b$	$0.02 \pm < 0.01^b$	—	0.33 ± 0.03^c	$0.04 \pm < 0.01^c$
Plasma								
Hep-gluc	0.33 ± 0.06^a	0.35 ± 0.06^b	$0.08 \pm < 0.01^b$	0.16 ± 0.02^a	$0.03 \pm < 0.01^b$	0.06 ± 0.01^a	1.57 ± 0.12^a	0.39 ± 0.04^a
Hep-BCAA	0.13 ± 0.01^b	0.50 ± 0.03^a	2.29 ± 0.39^a	0.05 ± 0.01^b	1.03 ± 0.19^a	$0.02 \pm < 0.01^b$	1.00 ± 0.08^b	$0.28 \pm 0.02^{b,c}$
PC-shunt	0.14 ± 0.01^b	0.20 ± 0.01^c	0.09 ± 0.01^b	$0.06 \pm < 0.01^b$	0.04 ± 0.01^b	$0.03 \pm < 0.01^b$	0.40 ± 0.04^c	$0.08 \pm < 0.01^c$
Sham-op	0.16 ± 0.01^b	0.18 ± 0.01^c	0.11 ± 0.01^b	$0.05 \pm < 0.01^b$	$0.05 \pm < 0.01^b$	$0.03 \pm < 0.01^b$	0.42 ± 0.04^c	$0.05 \pm < 0.01^c$

For other details see table I.

Table III. Regional indole amines and tryptophan 18 h after hepatectomy and in control rats (means $\pm$ SEM values)

	TRP ng/g $\times 10^{-3}$	Serotonin ng/g	5-HIAA ng/g
Cortex			
Hep-gluc	20.6 $\pm$ 1.5 ^a	541 $\pm$ 45 ^a	768 $\pm$ 79 ^a
Hep-BCAA	1.8 $\pm$ 0.2 ^d	267 $\pm$ 24 ^b	203 $\pm$ 22 ^c
PC-shunt	12.5 $\pm$ 0.9 ^b	526 $\pm$ 51 ^a	518 $\pm$ 58 ^b
Sham-op	7.7 $\pm$ 0.4 ^c	411 $\pm$ 50 ^a	323 $\pm$ 38 ^c
Mesencephalon			
Hep-gluc	14.6 $\pm$ 1.2 ^a	994 $\pm$ 60 ^a	2,056 $\pm$ 204 ^a
Hep-BCAA	2.0 $\pm$ 0.2 ^d	484 $\pm$ 43 ^b	387 $\pm$ 44 ^c
PC-shunt	9.1 $\pm$ 0.4 ^b	893 $\pm$ 22 ^a	1,546 $\pm$ 76 ^b
Sham-op	5.8 $\pm$ 0.4 ^c	851 $\pm$ 94 ^a	1,016 $\pm$ 130 ^c
Cerebellum			
Hep-gluc	39.3 $\pm$ 6.1 ^a	83 $\pm$ 4 ^a	427 $\pm$ 36 ^a
Hep-BCAA	3.1 $\pm$ 0.4 ^d	45 $\pm$ 7 ^b	99 $\pm$ 9 ^c
PC-shunt	19.6 $\pm$ 3.0 ^b	97 $\pm$ 6 ^a	272 $\pm$ 18 ^b
Sham-op	11.0 $\pm$ 1.6 ^c	89 $\pm$ 8 ^a	171 $\pm$ 10 ^c
Diencephalon			
Hep-gluc	17.4 $\pm$ 1.8 ^a	869 $\pm$ 133 ^a	1,518 $\pm$ 172 ^a
Hep-BCAA	1.8 $\pm$ 0.1 ^d	456 $\pm$ 54 ^b	398 $\pm$ 40 ^c
PC-shunt	12.0 $\pm$ 0.3 ^b	892 $\pm$ 16 ^a	1,509 $\pm$ 97 ^a
Sham-op	7.3 $\pm$ 0.2 ^c	951 $\pm$ 76 ^a	1,031 $\pm$ 68 ^b
Hypothalamus			
Hep-gluc	19.1 $\pm$ 1.5 ^a	1,370 $\pm$ 57 ^a	1,138 $\pm$ 72 ^a
Hep-BCAA	1.9 $\pm$ 0.2 ^d	599 $\pm$ 53 ^d	343 $\pm$ 26 ^d
PC-shunt	9.7 $\pm$ 0.5 ^b	1,207 $\pm$ 50 ^b	946 $\pm$ 75 ^b
Sham-op	6.4 $\pm$ 0.3 ^c	976 $\pm$ 63 ^c	546 $\pm$ 50 ^c
Pons medulla			
Hep-gluc	18.3 $\pm$ 1.3 ^a	900 $\pm$ 27 ^a	2,031 $\pm$ 114 ^a
Hep-BCAA	2.3 $\pm$ 0.3 ^d	398 $\pm$ 25 ^c	337 $\pm$ 35 ^d
PC-shunt	10.0 $\pm$ 0.4 ^b	834 $\pm$ 22 ^a	1,399 $\pm$ 49 ^b
Sham-op	6.4 $\pm$ 0.1 ^c	739 $\pm$ 26 ^b	771 $\pm$ 59 ^c
Hippocampus			
Hep-gluc	21.7 $\pm$ 1.4 ^a	454 $\pm$ 46 ^a	859 $\pm$ 57 ^a
Hep-BCAA	2.4 $\pm$ 0.3 ^d	216 $\pm$ 17 ^c	240 $\pm$ 24 ^d
PC-shunt	12.2 $\pm$ 0.3 ^b	430 $\pm$ 34 ^a	707 $\pm$ 36 ^b
Sham-op	6.6 $\pm$ 0.1 ^c	334 $\pm$ 16 ^b	474 $\pm$ 37 ^c
Striatum			
Hep-gluc	20.2 $\pm$ 1.6 ^a	598 $\pm$ 20 ^c	1,600 $\pm$ 110 ^a
Hep-BCAA	2.0 $\pm$ 0.2 ^d	304 $\pm$ 25 ^d	521 $\pm$ 71 ^d
PC-shunt	15.2 $\pm$ 0.4 ^b	775 $\pm$ 32 ^a	1,317 $\pm$ 70 ^b
Sham-op	8.9 $\pm$ 0.3 ^c	673 $\pm$ 35 ^b	929 $\pm$ 78 ^c

8 animals in each group.

For other details see table I.

Indoleamines and TRP in Brain (table III). The concentrations of TRP and 5-HIAA in all regions, except in the diencephalon and cortex for 5-HIAA, could be ranked according to groups A>C>D>B. Compared to group D, group C concentrations of serotonin were higher in the striatum, hippocampus, pons medulla and hypothalamus. The concentrations of serotonin were higher in the hypothalamus and lower in the striatum in group A than in group C. The concentrations of serotonin were lowest in group B in all regions.

Discussion

Altered neurotransmitter metabolism has been suggested to contribute to hepatic encephalopathy. Local variation in neurotransmitter metabolism [4, 5, 16] may be of importance, and this experiment was designed to study the amino acid and indole amine concentrations in eight regions of the brain in hepatectomized rats after glucose and BCAA infusion, and after glucose infusion alone.

In the different brain regions of hepatectomized rats infused with glucose, the accumulation of LNAA, except for the BCAA, was notable (table I, II). Similar results have previously been reported in half brains of hepatectomized rats [14]. Comparing hepatectomized rats receiving glucose to those receiving glucose and BCAA, the release of ALA and GLN from peripheral tissues was greatly stimulated by the BCAA while the release of PHE, TYR, MET and HIS was inhibited. This may be in agreement with the anticatabolic effect on protein metabolism of BCAA, that has been reported from *in vitro* experiments with muscle preparations and from studies in humans [8-10, 18]. Increased alanine and glutamine levels after BCAA infu-

sion have also been reported in humans after BCAA treatment [1, 18]. High plasma concentrations of GLN may be consistent with the hypothesis that BCAA accelerate ammonia detoxification in the muscles [12].

It has been proposed that in acute hepatic failure the blood-brain barrier does not remain intact [17]. However, this experiment showed that in hepatectomized rats infused with glucose and BCAA compared to hepatectomized rats infused with glucose alone, the concentrations of TYR, HIS, TRP and MET decreased more in the brain than in the plasma. These findings suggest that, with respect to competition for transport among the LNAA, the blood-brain barrier seems to keep its integrity.

In hepatic failure in animals and man, increased brain [2, 4, 5, 16] and CSF [19] levels of TRP, serotonin and 5-HIAA have been found, suggesting an accelerated serotonin turnover which may contribute to hepatic coma.

In hepatectomized rats BCAA infusion reduced the TRP levels in the brain below normal levels, and simultaneously the concentrations of serotonin and 5-HIAA were sharply reduced in all regions of the brain (table III). These observations clearly show the capability of the BCAA to reduce the brain TRP concentrations and thereby depress the indole amine metabolism. The clinical implication of these findings on hepatic encephalopathy is controversial and demands further investigation [3, 8, 20, 21].

In rats with liver cirrhosis, infusion of BCAA could prevent ammonia-induced coma, whereas this effect could not be seen in rats with acute liver damage after carbon tetrachloride injection [13]. Similarly, in the present study with hepatectomy as a model of acute hepatic failure, increased survival

could not be demonstrated after BCAA infusion.

The results of our study could be summarized as follows: (1) even in a severe model as total hepatectomy, infusion of BCAA managed to reduce the efflux of amino acids from peripheral tissues; (2) infusion of BCAA sharply reduced the brain concentrations of other neutral amino acids and indole amines in all brain regions; (3) there were regional differences in the brain concentrations of the amino acids and indole amines, but the response to the different treatments was similar in all regions, and (4) low TRP concentrations in the brain regions were followed by depressed indole amine metabolism throughout the brain.

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EFFECTS OF INFUSING BRANCHED CHAIN AMINO ACIDS AND AMMONIUM SALTS
IN RATS AFTER PORTACAVAL ANASTOMOSIS

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ABSTRACT

During infusion into rats with a portacaval shunt of either ammonium-salts alone or ammonium-salts combined with the three branched chain amino acids (BCAA) in equimolar quantities, we assessed neurologic function and measured plasma and brain ammonia and amino acids and the brain content of amine neurotransmitters and their metabolites. Survival was lengthened and neurologic function was preserved longer in rats receiving BCAA. Infusion of BCAA resulted in lower plasma and brain ammonia concentrations as compared with rats receiving ammonium-salts alone. Plasma glutamine and alanine were higher in rats receiving BCAA, suggesting increased ammonia detoxification. Loss of neurologic function, regardless of infusion, eventually occurred and corresponded with decreased brain norepinephrine (NE) and increased brain alanine. These results suggest that BCAA can protect against hyperammonemia by stimulating the peripheral detoxification of ammonia.

In recent years considerable interest has developed regarding the administration of branched chain amino acids (BCAA) in the treatment of hepatic coma. The clinical efficacy as well as the mode of action of this therapy are still undetermined. In two randomized studies,^{1,2} BCAA were demonstrated to be better than or at least equivalent to standard forms of therapy for hepatic coma, such as lactulose or neomycin, which are intended to lower blood ammonia levels. However, another study failed to demonstrate any advantage of BCAA versus placebo therapy.³ It is possible that these discrepancies reflect either differences in patient populations, lack of objective measures of hepatic encephalopathy, or differences in dose and route of administration of amino acids and caloric support.

One effect of BCAA which may be therapeutically beneficial is the correction of the abnormal plasma and brain amino acid pattern characteristic of hepatic encephalopathy. Low plasma concentrations of BCAA and high levels of the aromatic amino acids, tyrosine, phenylalanine and tryptophan are frequently observed in patients with liver cirrhosis and hepatic encephalopathy.⁴ These changes, together with an increased rate of blood-brain barrier transport of neutral amino acids,^{5,6} may increase the influx of aromatic amino acids to the brain, thereby affecting neurotransmitter metabolism and altering neurological function. BCAA administration may reduce the brain content of aromatic amino acids either indirectly by promoting muscle protein synthesis⁷ and thereby lowering plasma aromatic amino acid levels, or directly by competing with the aromatic amino acids for transport at the blood-brain barrier.⁸

However, other effects of BCAA not directly related to aromatic amino

acid metabolism could also be important. Thus, BCAA stimulate glutamine synthesis in muscle both in vitro⁹ and in vivo,¹⁰ a reaction which may assume greater than normal importance in the detoxification of ammonia in presence of liver failure.¹¹ Recently, Hayashi et al¹² have reported that intraduodenal prefeeding of a BCAA-rich amino acid solution prevented encephalopathy caused by injection of ammonium acetate in rats with CCl₄-induced liver cirrhosis. In the present study, we infused solutions containing either ammonium salts alone or both ammonium salts and BCAA into rats with portacaval shunts. The effect of these infusions on neurological function and survival as well as blood and brain concentrations of ammonia, amino acids and neurotransmitters was studied.

MATERIAL AND METHODS

Male Sprague-Dawley rats weighing 300-350 g were used. End-to-side portacaval shunts were constructed according to the technique of Lee and Fisher.¹³ Eight days after the operation, a silastic catheter for infusions was positioned in the external jugular vein and was protected by a flexible metal sheath attached to a swivel. Animals were anesthetized with diethyl ether for all surgical procedures.

Two separate experiments were performed.

In the first experiment, 11 rats were infused with a solution containing 0.4 mole/l NH_4^+ -salts (200 mmole/l NH_4^+ acetate + 200 mmole/l NH_4^+ bicarbonate, pH 7.4) and 11 rats were infused with 0.4 mole/l NH_4^+ -salts + 0.3 mole/l BCAA (0.1 mole/l each leucine, isoleucine and

valine; final pH, 7.4). The rate of infusion was 0.9 ml/100 g body weight per hr. The infusion was continued until the rat lapsed into coma and died. Neurological evaluation was assessed hourly during infusion. The 15-point neurological assessment battery consisted of testing the following reflexes,¹⁴ each with an assigned numerical value.

Flexion Reflex: Each foot was pinched with a tissue forceps. Withdrawal of a foot indicated a positive response. With a value of 1 being assigned for each positive response, a total score of 4 was possible.

Grasping Reflex: The rat was held by the loose skin of the back and a small metal rod was touched to the surface of both palms at once. If the rat grasped the rod with the forepaws, a positive grasping reflex was inferred and a value of 1 was scored.

Righting Reflex: The rat was placed on its back and a score of 1 was assigned if it immediately righted itself. The rat was then held at the lumbar area and the body was tilted to the left and then to the right. If the rat's head moved in the opposite direction of the body tilt, a value of 1 was scored for each response.

Placement Reflex: The rat was slowly moved in the anterior direction toward the edge of a table. If the forepaws were placed on the table as the rat approached, a value of 1 was assigned for each correctly placed forepaw (maximum, 2). The rat was placed on the table with the body axis parallel to the edge of the table. If a hindlimb that was pulled down over the table's edge was immediately replaced on the table, a value of 1 was scored for each hindlimb. The rat was suspended by its tail until the vibrissae touched the edge of the table. If the forepaws were extended to touch the

table, a score of 1 was assigned.

Corneal Reflex: One eye of the rat was lightly touched with a cotton-tipped swab. If an eye-blink reflex immediately followed, a score of 1 was entered on the data sheet.

Head-Shaking Reflex: Air was blown into the ear using a small tube. If head shaking occurred in response to this stimulus, a score of 1 was given.

In the second experiment, 12 rats were infused with 0.4 mole/l NH_4^+ -salts (Group A) and another 12 rats received 0.4 mole/l NH_4^+ -salts + 0.3 mole/l BCAA (Group B) at the same rate as above. The neurological score was assessed hourly as before. However, half of the rats in groups A and B were killed when their score indicated minimal neurological impairment (score 14-13; about 3 hr infusion time for Group A and about 6 hr for Group B) and the remaining rats in each group were sacrificed when their score indicated severe neurological impairment (score < 5; about 6 hr infusion time for Group A and about 10 hr for Group B).

Blood and Brain Sampling

Rats were anesthetized with ether and 3 ml of blood was collected immediately from the inferior caval vein. The skull was exposed through a midline incision and an open-bottom plastic cup was clamped tightly over the calvarium. Liquid nitrogen was poured into the plastic cup and the brains were allowed to freeze in situ for 3 min, during which time the rats were alive and breathing. The animals were then decapitated and the head was completely frozen by immersion in liquid nitrogen.

Brain Dissection

The heads were allowed to warm to about -5°C in a freezer and the brains were removed from the skull. One hemisphere was dissected for subsequent assay of neurotransmitters and metabolites. The other hemisphere was dissected into roughly two equal portions; the anterior half (mostly cortex and striatum) was processed for amino acid analysis, while the posterior half was processed for ammonia assay.

Extractions and Analytic Procedures

Amino acid analyses

The plasma was separated by centrifugation and kept frozen at -72°C until analyzed. Plasma and brain amino acids were determined by a Beckman 121-MB amino acid analyzer with automatic integration. Plasma was deproteinized by mixing 0.5 ml of plasma with 1.5 ml of 5% (wt/vol) sulfosalicylic acid, pH 1.7 containing 133 nmol/ml thienylalanine as internal standard. After vortexing, the samples were centrifuged ($30,000 \times g$, 4°C) and passed through a 0.45 micron Millipore filter. Brain tissue was homogenized in three times its weight of 5% (wt/vol) sulfosalicylic acid, pH 1.4 containing 133 nmol/ml thienylalanine as above. Fifty microliters of the supernatant of each brain homogenate were analyzed. To accurately quantitate glutamine and glutamate, the supernatant of the brain homogenate was further diluted 1:25 with 0.15 mole/L Li citrate buffer, pH 2.2 and analyzed in a special short analysis program.

Ammonia assay

Plasma was deproteinized by addition to a tube containing an equal

volume of frozen 0.5 mole/L HClO_4 to which an additional volume of ice-cold 0.5 mole/L HClO_4 was then added and the samples homogenized with a Polytron homogenizer (Brinkman Instruments). After centrifugation ($10,000 \times g$, 15 min, 4°C), the supernatant was neutralized to pH 7.0-7.5 by addition of 2.0 mole/L KHCO_3 ; precipitated KClO_4 was removed by centrifugation ($10,000 \times g$, 15 min, 4°C). Brain tissue (0.4-0.7 g) was weighed while frozen and transferred to tubes containing 0.2 ml of frozen 0.5 mole/L HClO_4 . An additional 2.0 ml of ice-cold 5 mmole/L EDTA was added to each tube and the mixture homogenized for 10 s (on ice). Homogenates were further treated to yield neutralized extracts as described above.

Ammonia was measured using glutamate dehydrogenase (E.C.1.4.1.3) in a reaction mixture containing in 1.0 ml 0.25 mole/L Tris buffer, pH 8.0, 2 mmole/L α -ketoglutarate, 0.16 mmole/L NADPH and 4.3 units of glutamate dehydrogenase. Neutralized sample extract was added in amounts (0.1-0.5 ml) depending on expected ammonia content and, when necessary, deionized water was added to bring the final volume to 1.0 ml. The reaction was initiated by addition of sample and the mixture was incubated at room temperature for 60 min, at which time the absorbance at 340 nm was measured. A blank from which enzyme was omitted was prepared for each sample.

Assay of Neurotransmitters and Metabolites

Hemisphere levels of serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and norepinephrine (NE) were assayed by high performance liquid chromatography with electrochemical detection, according

to a modification of the method of Loullis et al.¹⁵ Each sample was homogenized in 10 volumes of 1 mole/L formic acid:acetone (15/85, V:V) that contained N-methyldopamine (NMeDA) as an internal standard. After centrifugation (10,000 x g, 10 min, 4°C), the supernatants were divided into two 2.5 ml aliquots and 7.5 ml of heptane:chloroform (8/1, V:V) was added to each sample. The samples were next shaken (10 min) and centrifuged (2,500 x g, 10 min 4°C), with the organic phase being discarded. In one set of samples, DA and NMeDA were adsorbed onto 100 mg of activated alumina, washed twice with distilled water (10 ml) and eluted from the alumina with 0.1 N HCl. Twenty μ l of each of these samples were injected onto the column to separate and quantify NE, NMeDA and DA. The aqueous phase of the other set of samples was dried under a stream of nitrogen, with the sample being reconstituted in 0.8 ml of the mobile phase buffer (10% methanol and 90% of 0.1 mole/l KH_2PO_4 , 0.185 mmole/l sodium octylsulfate, 0.195 mmole/l EDTA; pH 3.15).

Statistical Analysis

Significance of differences between groups was determined by two-way ANOVA followed by Scheffe's t-test (ammonia data) or by Student's t-test (amino acid and neurotransmitter data).

RESULTS

Experiment 1

The number of rats surviving after increasing lengths of infusion and their hourly neurological scores are reported in Figure 1. It is evident

that survival was longer and that deterioration of neurological function was slower in rats infused with NH_4^+ -salts + BCAA in comparison to rats infused with NH_4^+ -salts only. In both groups some rats convulsed before death.

Experiment 2

Ammonia

As previously observed, rats infused with NH_4^+ -salts alone showed a mild but measurable degree of neurological impairment after about 3 hr of infusion, whereas the rats infused with NH_4^+ -salts + BCAA required a considerably longer infusion time (about 6 hr) to develop the same neurological deficit. Plasma and brain ammonia levels were not significantly different between these two groups (Table 1). Compared to another group ($n=6$) of rats eight days after portacaval shunt but otherwise untreated, plasma and brain ammonia levels in the infused rats with slight neurological impairment were elevated 4- to 5-fold (untreated, 8 days after portacaval shunt; plasma: 200 ± 14 nmol/ml; brain: 383 ± 29 nmol/g, $\bar{x} \pm \text{SEM}$).

As observed in Experiment 1, rats infused with NH_4^+ -salts + BCAA required a longer infusion period (about 10 hr), compared to rats receiving NH_4^+ -salts alone, to reach a state of severe neurological impairment. However, although they displayed similar neurological deficits, rats receiving NH_4^+ -salts + BCAA had significantly lower plasma and brain ammonia levels compared to rats receiving NH_4^+ -salts alone. Among rats receiving NH_4^+ -salts alone, those with low neurological scores had significantly higher plasma and brain ammonia levels than those with high neurological

scores which were infused for a shorter length of time. In contrast, among rats receiving NH_4^+ -salts + BCAA, deterioration of neurological function was not associated with a significant change in plasma or brain ammonia concentrations. The highest concentrations of ammonia, both in plasma and brain, were observed in rats receiving NH_4^+ -salts only, even though the total infused dose of NH_4^+ -salts in rats receiving NH_4^+ -salts + BCAA was the same or greater.

Plasma Amino Acids

Compared to rats receiving NH_4^+ -salts only, rats infused with NH_4^+ -salts + BCAA had markedly elevated plasma concentrations of leucine, isoleucine and valine, as expected. Infusion of BCAA was associated with lower plasma levels of lysine, methionine, phenylalanine, tyrosine, tryptophan and threonine, regardless of the infusion time. In contrast, rats receiving NH_4^+ -salts + BCAA had higher plasma levels of alanine and glutamine.

Brain Amino Acids

Addition of BCAA to the infusion solution increased brain BCAA levels and greatly decreased brain levels of histidine, methionine, phenylalanine, threonine, tryptophan and tyrosine. The only amino acids in this group which differed in concentration between the groups with high and low neurological scores were the BCAA themselves, although their concentrations were markedly lower in rats receiving NH_4^+ -salts alone.

Among the non-essential amino acids, only brain alanine showed a consistent significant rise over the period of infusion, which was correlated to the neurological state of the rats. Glutamine was extremely elevated in all groups.

Neurotransmitters and Metabolites

Hemisphere levels of NE were lower than normal in all groups and were lowest in rats with lowest neurological scores, regardless of treatment. DA and HVA concentrations did not show any consistent relationship either to neurological score or to treatment. In contrast, the level of DOPAC, another DA metabolite, was consistently higher in rats with low neurological scores. The levels of 5-HT and 5-HIAA were decreased in rats receiving NH_4^+ -salts + BCAA compared to those receiving NH_4^+ -salts only; however, 5-HT and 5-HIAA levels showed no consistent relationship with neurological score.

DISCUSSION

In the present studies, we investigated the effect of continuous infusion of ammonium-salts in portacaval-shunted rats, which are particularly sensitive to ammonia administration.¹⁶ In studies by others, ammonia or other coma-producing substances are usually given by single, intraperitoneal injection which produces rapid changes in the animals' behavior.^{12,16,17} In one study,¹⁷ normal rats injected with ammonium acetate lost muscle control within five minutes, then hyperventilated and lapsed into coma. Such rapid deterioration may not be related to clinical hepatic coma, in which ammonia levels are elevated for longer time periods. That continuous ammonia infusion may provide a more physiologic model of hyperammonemia is suggested by the gradual loss of neurologic function over several hours, which was observed in the present study. Moreover, the use of the neurologic test battery in the present studies provided a means of

assessing the degree of encephalopathy with some measure of objectivity. The combination of continuous infusion and neurologic testing permitted a clear demonstration that the addition of BCAA to ammonium-salts prolonged survival and delayed neurologic deterioration.

The remainder of the study attempted to identify specific factors which were affected by the BCAA infusion and which were correlated with the neurologic state. Infusion of BCAA prevented the steady rise in plasma ammonia concentration seen in the group receiving ammonium-salts alone (Table 1). In rats receiving both ammonium-salts and BCAA, plasma ammonia was not higher after 10 hr of infusion than after 6 hr, suggesting that peripheral metabolism of ammonia was able to keep pace with the infusion rate ($3.6 \text{ mmol} \times \text{kg}^{-1} \times \text{hr}^{-1}$). In contrast, in rats receiving ammonium-salts only, plasma ammonia levels rose in proportion to the length of infusion. Similarly, brain ammonia in rats receiving only ammonium-salts doubled as infusion time doubled; whereas in the group also receiving BCAA, brain ammonia rose to a lesser degree and remained significantly less than in the rats receiving ammonia alone.

In comparison to amino acid concentrations previously reported from this laboratory¹⁸ in plasma of rats with a portacaval shunt and during an infusion of only glucose and electrolytes, infusion of ammonium-salts in the present studies resulted in a similar pattern (Table 2), with the most obvious differences being increased glutamine and arginine in the latter group. Coinfusion of BCAA and ammonium-salts resulted in a marked increase in plasma levels of glutamine and alanine and decreased levels of tyrosine and the essential amino acids lysine, methionine, phenylalanine, threonine

and tryptophan. This decrease in essential amino acid concentrations during infusion of BCAA is similar to that previously reported in rats following portacaval shunt and total hepatectomy¹⁸ and may reflect increased protein synthesis and decreased protein breakdown in peripheral tissues. Infusion of BCAA and glucose into hepatectomized rats which are also hyperammonemic was found to greatly stimulate the rise in plasma glutamine and alanine concentrations. Taken together with the present results, these observations suggest that BCAA facilitate the incorporation of ammonia into glutamine and alanine by peripheral tissues. One explanation of this effect may be that glutamine synthesis from glutamic acid and ammonia becomes limited by the availability of glutamate when tissue ammonia levels remain elevated for extended periods. Infused BCAA may stimulate transamination of alpha-ketoglutarate, thus assuring replenishment of glutamate lost to glutamine synthesis. The rise in alanine levels may be due to enhanced glutamate-pyruvate transamination, with subsequent alanine release, or to synthesis of alanine by intestine, which normally takes up glutamine from the blood and releases alanine.¹⁹ The ability of BCAA to delay the rise in plasma ammonia may thus have been due to stimulation of ammonia incorporation into glutamine.

Infusing BCAA resulted in markedly lower brain concentrations of histidine, methionine, phenylalanine, threonine, tryptophan and tyrosine compared to rats receiving ammonium-salts alone. This result is not surprising since the latter amino acids and the BCAA together comprise the group of large neutral amino acids which share a common transport system across the blood-brain barrier.⁸ Therefore, raising the blood

concentrations of the BCAA to a high level should inhibit the uptake by brain of the other competitors for transport. Similar results were obtained in this laboratory when hepatectomized rats were infused with a solution containing BCAA.¹⁸

Neurologic status did not correlate with brain levels of any of the large neutral amino acids, suggesting that in this model of hyperammonemia, brain neutral amino acid levels do not alone determine production of coma. The amino acids in brain which correlated best with neurological status were alanine and glutamate, which rose and fell, respectively, as neurologic score decreased in both groups.

No obvious correlation existed between neurologic function and brain levels of 5HT, 5HIAA, DA and its metabolite HVA. Increased brain levels of the DA metabolite DOPAC were observed in both treatment groups as neurologic function declined. Increased formation of DOPAC from labeled L-dihydroxy-phenylalanine administered to hepatectomized rats has been previously reported.²⁰

Brain content of the biogenic amines NE, DA and 5HT has previously been studied in rats in hepatic coma after total hepatectomy.^{18, 21} In the present studies, both the percentage decrease in brain NE content over time and the inverse correlation between brain NE and brain alanine were similar to what was observed in rats after total hepatectomy.¹⁸ Taken together, these results suggest that extreme hyperammonemia is sufficient to reduce brain NE levels, and it is therefore unnecessary to postulate a role for additional factors which might accumulate after total hepatectomy. Although the present studies strengthen the association between loss of neurologic

function and decreased brain NE content, they do not permit speculation on the relationship between brain ammonia and brain NE. It is not unreasonable to suggest, however, that BCAA infusion may have preserved neurologic function by delaying the rapid accumulation in brain of ammonia and thus preventing decline in brain NE below levels necessary for consciousness.

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Fig. 1 Effect of infusing ammonium-salts alone or combined with BCAA on neurological function, tested hourly, and on survival after 4, 8 and 12 hours of infusion.

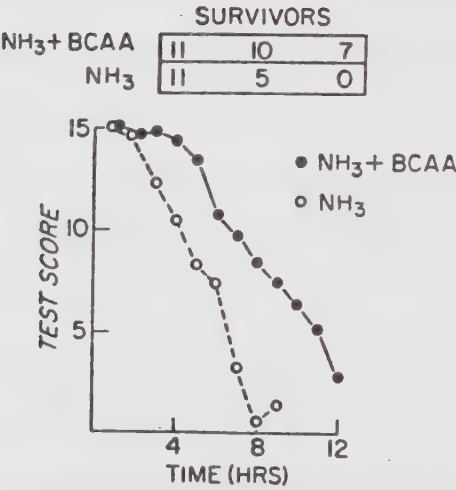


Table 1. Concentrations of Ammonia after Infusion of NH_4^+ -Salts (Group A)
or NH_4^+ -Salts + BCAA (Group B)

	Group A (NH_4^+ alone)		Group B (NH_4^+ + BCAA)	
	n =			
Neurological score	6	6	6	6
	13-14	< 5	13-14	< 5
Approximate length of infusion (hrs)	3	6	6	10
Plasma (nmol/ml)	1033 $\pm$ 78	2064 $\pm$ 179*	995 $\pm$ 81	853 $\pm$ 98
Brain (nmol/g)	1838 $\pm$ 191	3671 $\pm$ 466*	1593 $\pm$ 125	2070 $\pm$ 132

Concentrations are $\bar{X} \pm \text{S.E.M.}$ for groups of 6 rats.

* $p < 0.01$; significantly different from all other values in same row; no other comparisons were statistically significant.

Table 2. Concentrations of Amino Acids in Plasma after Infusion of NH_4^+ -Salts (Group A) or NH_4^+ -Salts + BCAA (Group B)

	Group A (NH ₄ ⁺ alone)		Group B (NH ₄ ⁺ + BCAA)		
Approximate infusion time (hrs)	3	6	6	10	Comparison values [†]
Amino Acids	(nmol/ml)				
Essential					
HIS	84 ± 3	88 ± 6	85 ± 5	79 ± 4	80 ± 5
ILE	25 ± 2	33 ± 7	869 ± 85	993 ± 105	30 ± 1
LEU	51 ± 4	64 ± 7	991 ± 91	1126 ± 101	68 ± 4
LYS	255 ± 10	282 ± 13	111 ± 10	139 ± 8	275 ± 15
MET	28 ± 3	25 ± 2	11 ± 1	20 ± 3	32 ± 2
PHE	79 ± 4	78 ± 4	34 ± 2	34 ± 3	93 ± 6
THR	80 ± 4	91 ± 5	37 ± 3	43 ± 5	84 ± 6
TRP	55 ± 9	38 ± 8	27 ± 4	22 ± 3	---
VAL	51 ± 4	67 ± 8	1408 ± 123	1581 ± 84	80 ± 4
Non-Essential					
ALA	220 ± 16	219 ± 31	305 ± 21	450 ± 86	271 ± 31
ARG	141 ± 16	149 ± 17	170 ± 9	222 ± 6	99 ± 5
GLU	29 ± 2	28 ± 3	51 ± 6	79 ± 14	52 ± 4
GLN	1010 ± 29	1034 ± 102	2042 ± 139	2427 ± 211	636 ± 31
GLY	211 ± 6	220 ± 20	164 ± 11	168 ± 17	243 ± 14
SER	127 ± 7	111 ± 12	114 ± 11	127 ± 12	142 ± 9
TYR	49 ± 9	53 ± 4	23 ± 2	25 ± 2	67 ± 4

Concentrations are $\bar{x} \pm \text{S.E.M.}$ for groups of 6 rats.

† from ref #18

Table 3. Concentrations of Amino Acids and Amine Neurotransmitters in Brain after NH_4^+ -Salts (Group A) or NH_4^+ -Salts + BCAA (Group B)

Approximate infusion time (hrs)	Group A (NH ₄ ⁺ alone)		Group B (NH ₄ ⁺ + BCAA)		Comparison values [†]
	3	6	6	10	
(nmol/g)					
Amino Acids					
Essential					
HIS	322 ± 18	315 ± 18	50 ± 6	48 ± 4	178 ± 9
ILE	50 ± 7	98 ± 8***	268 ± 20	328 ± 21*	12 ± 1
LEU	109 ± 7	191 ± 12***	332 ± 17	432 ± 25**	56 ± 3
LYS	280 ± 11	261 ± 4	205 ± 10	194 ± 12	294 ± 15
MET	77 ± 6	87 ± 5	15 ± 4	9 ± 1	58 ± 1
PHE	264 ± 20	251 ± 8	17 ± 2	18 ± 2	153 ± 12
THR	545 ± 23	520 ± 57	200 ± 21	177 ± 21	455 ± 28
TRP	60 ± 7	66 ± 3	33 ± 2	32 ± 2	29 ± 1
VAL	95 ± 8	137 ± 8***	365 ± 21	513 ± 29**	64 ± 2
Non-Essential					
ALA	1381 ± 60	2082 ± 89***	921 ± 57	1720 ± 176**	531 ± 9
ARG	129 ± 7	118 ± 4	174 ± 7	167 ± 8	112 ± 5
GLU	9178 ± 246	8359 ± 330	9595 ± 303	8385 ± 193**	11816 ± 190
GLN	25212 ± 1212	30906 ± 885**	25247 ± 550	27291 ± 702*	10198 ± 918
GLY	971 ± 42	1070 ± 25	1081 ± 27	1157 ± 27	1191 ± 29
SER	805 ± 19	601 ± 51	625 ± 22	652 ± 13	812 ± 20
TYR	224 ± 18	202 ± 10	17 ± 2	16 ± 1	136 ± 10
Neurotransmitters and Metabolites (ng/g)					
NE	283 ± 16	228 ± 11*	310 ± 22	190 ± 11***	410 ± 10
DA	698 ± 14	720 ± 28	757 ± 42	677 ± 15	820 ± 30
DOPAC	163 ± 11	210 ± 10*	113 ± 2	214 ± 19***	---
5-HT	726 ± 23	507 ± 17***	318 ± 12	330 ± 14	913 ± 40 [‡]
5-HIAA	1187 ± 111	898 ± 53*	282 ± 9	310 ± 16	882 ± 44 [‡]
HVA	114 ± 5	95 ± 13	56 ± 7	80 ± 11	---

Concentrations are $\bar{x} \pm \text{S.E.M.}$ for groups of 6 rats.

Significantly different from shorter infusion time in same group,

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

[†] from ref #18; [‡] from ref #21



The effects on the plasma concentrations of amino acids, insulin and glucagon and on the CSF concentrations of amino acids and indoleamines after administration of ammonia to patients with liver cirrhosis

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ABSTRACT

Infusion of ammonium salts in rats increases the brain concentrations of neutral amino acids. To study the effect of ammonia administration on CSF and plasma amino acids in humans we performed 8 ammonia tolerance tests in 7 patients with stable liver cirrhosis and a history of encephalopathy. The grade of encephalopathy was determined by psychometric tests. The plasma and CSF ammonia concentrations were greatly elevated after the ammonia intake. However, the CSF glutamine concentrations increased only in one patient who exhibited a pronounced encephalopathy. The glutamine concentrations in the cerebrospinal fluid (CSF) correlated well with the concentrations of phenylalanine ($r=0.90$), tyrosine ($r=0.90$) and methionine ($r=0.92$). The plasma concentrations of the branched chain amino acids decreased or remained unchanged in all patients except in one. No correlation between plasma ammonia concentrations and plasma glucagon and insulin concentrations was found. It was not demonstrated that ammonia administration increases the concentrations of the neutral amino acids in the CSF of patients with liver cirrhosis with slight or no hepatic encephalopathy (HE) within six hours. The good correlation between glutamine and the other neutral amino acids may support the hypothesis that glutamine plays a role in the brain concentrations of the other LNAA.

INTRODUCTION

In liver failure, high glutamine concentrations in the central nervous system, secondary to hyperammonemia, have been suggested to raise the concentrations of the neutral amino acids in brain and thereby disturb the neurotransmitter metabolism and contribute to hepatic encephalopathy (HE) (1). In support of this assumption, infusion of ammonium salts in experimental animals has been demonstrated to increase the brain uptake and the brain concentrations of neutral amino acids (2,3). If ammonia has the same effect on patients with liver disease remains to be shown.

The typical plasma neutral amino acid changes in liver failure comprise a decrease in the branched chain amino acids and an increase in phenylalanine, tyrosine, methionine and free tryptophan (4,5). Alterations in plasma amino acids may be mediated by changes in pancreatic hormones. Thus, hyperinsulinemia in liver failure has been suggested to reduce the plasma BCAA concentrations (6) but this has not been confirmed by other investigators (7). A connection between the peripheral metabolism of ammonia and that of BCAA has been suggested in in vitro studies on rats (8,9) and in studies on patients with liver cirrhosis (10) after an ammonia load.

Hyperammonemia in patients with liver disease has also been proposed to contribute to high plasma concentrations of glucagon and insulin (10).

In the present investigation we performed ammonia tolerance tests on patients with liver cirrhosis to study 1/ the effect on the concentrations of the neutral amino acids and indoleamines in CSF 2/ the effect on the concentrations of neutral amino acids, insulin and glucagon in plasma.

MATERIALS AND METHODS

Patients

The study was carried out on seven male cirrhotic patients. All of them had portal hypertension and a history of previous bleedings from oesophageal varices. All patients except one had a mesocaval shunt. The diagnosis was based on clinical, laboratory and roentgenologic findings and was always confirmed by a liver biopsy. The patients had had episodes of encephalopathy. They were regularly tested by a clinical psychologist and the

tests were performed within a month preceding the ammonia tolerance test. The psychometric tests utilized in this study are in general use at the Department of Psychiatry,

University of Lund, to diagnose diffuse organic brain dysfunction. The test battery was designed to evaluate visual memory and cognitive motor functions. Lowered performance of these tests can reflect a slowing of general response, concentration difficulties and fatigue typical of diffuse brain dysfunctions (11,12,13). The following tests were used to assess encephalopathy:

Visual Retention Test (Benton): Test of conceptual memory, visual perception abilities and memory for geometrical forms.

Perceptual speed (Digit Symbol Test): A visual motor test that tests the capacity to code symbols with digits.

Dot Test (Bourdon Wiersma): This test is very sensitive to concentration difficulties, lack of tenacity and to fatigue.

Colour Word Test: It is used to assess cognitive flexibility.

Manual Dexterity (cylinder board): Tests the capability to combine visual perception and hand motor function.

Trailmaking Test: Tests the cognitive motor abilities.

If a patient in one tested cerebral function deviates more than two standard deviations, he is considered to have slight HE. If he deviates in two functions or more, he is considered to have pronounced HE. The test results are corrected with regard to age and intellectual capacity.

The patients were well informed of the nature, the purpose and the possible risks involved in the study and they had given their written consent before the study. The protocols were approved by the Ethical Committee of the Medical Faculty, University of Lund.

Clinical data on the patients are given in table 1.

Ammonia Tolerance Test

The patients were fasted over night. Tests were started in the morning. Ammonium chloride

was dissolved in a glass of water. Five patients received ammonium chloride (2g) at the beginning of the test and another dose after 90 minutes. CSF (lumbar) samples were taken before and 180 minutes after administration of ammonium chloride. Three patients received ammonium chloride (2g) at the beginning of the test and after 90, 180 and 270 minutes. CSF was collected before the start of the test and after 360 minutes. Blood samples were drawn from a peripheral vein before administration of each dose of ammonium chloride and 30 minutes thereafter and at the end of the tests before taking the CSF samples. Plasma concentrations of amino acids (glutamine, glutamic acid, alanine, leucine, valine, isoleucine, phenylalanine, tyrosine, methionine), ammonia, insulin, glucagon and glucose were determined. In CSF, the concentrations of amino acids, 5-hydroxyindoleacetic acid (5-HIAA), 5-hydroxytryptamine (5-HT), 5-hydroxytryptophan (5-HTP), and ammonia were analyzed.

Quantitative determination of amino acids in plasma, deproteinized by sulfosalicylic acid, was carried out by a column chromatographic technique using a Biotronik LC 5000 automatic amino acid analyzer with a lithium buffer system.

Glucose was determined by the glucose oxidase technique.

Ammonia was determined with an enzymatic method using glutamate dehydrogenase.

The insulin and glucagon concentrations were measured with radioimmunoassay according to Heding (14) and von Schenck (15) respectively.

Indole amine assay: 5-Hydroxytryptophan (5-HTP), 5-Hydroxytryptamin (5-HT), and 5-Hydroxyindoleacetic acid (5-HIAA) were measured in the CSF by high performance liquid chromatography (HPLC) with electrochemical detection.

After addition of perchloric acid (0.4 M), the CSF samples were centrifuged at $34\,000 \times g$ for 10 min, and the supernatant was then filtered. For each analysis of 5-HTP, 5-HT, and 5-HIAA performed in the same run, 0.5 ml of this filtrate was placed in the automatic injector after addition of 100 ng α -methyl 5-HT as an internal standard. 10 μ l 5% L-cysteine solution was added to the filtrate as an antioxidant for 5-HIAA.

Statistics: Correlations were tested by Pearson product-moment correlation.

RESULTS

The basal plasma ammonia concentrations in the patients with mesocaval shunt were all above normal (Fig.1). Administration of ammonium chloride resulted in greatly elevated ammonia concentrations in plasma and in CSF, except for one patient who had not been operated upon with a mesocaval shunt. The basal plasma glutamine concentrations were elevated in only two patients (Fig. 2). Administration of ammonia resulted in a marked increase of plasma glutamine concentrations only in patient number 1 and 4 (Fig. 2). This rise of the glutamine concentrations was not accompanied by a decrease of the plasma alanine concentrations (Fig.2). The ammonia load reduced the plasma concentrations of glutamic acid in six tests but the changes were very small (Fig. 3). The CSF glutamine increased greatly only in patient number 5 who was also the most encephalopathic (Fig 4). This increase of glutamine in the CSF was associated with an increase of the other large neutral amino acids (LNAA) (Fig. 5). The basal plasma concentrations of the BCAA were not reduced compared to reference values*, whereas the basal plasma concentrations of phenylalanine, tyrosine, and methionine were all above normal levels (Fig. 6). Administration of ammonium chloride tended to increase the plasma concentrations of BCAA only in patient number 4. In the other patients, the BCAA were slightly reduced or remained unchanged after the ammonia load. The etiology of the liver cirrhosis of patient number 4 was sarcoidosis while that of the other patients was alcoholic cirrhosis. After the ammonia load, the plasma concentrations of phenylalanine, tyrosine and methionine were reduced or remained unchanged except in patient number 4. This patient showed a slight increase in the plasma concentrations of phenylalanine and tyrosine.

A good linear correlation was observed between the glutamine concentrations in CSF and the concentrations of phenylalanine, tyrosine and methionine, while the linear correlation between glutamine and the BCAA was poor (Table 2).

The linear correlation between the CSF concentrations of 5-HT and 5-HTP and the concentrations of glutamine in CSF was good (Table 2) (Fig. 7).

No correlation was observed between the insulin and glucagon concentrations in plasma and the plasma concentrations of ammonia. There was an increase in plasma glucagon after the ammonia load only in patient number 4 (Fig. 8).

The glucose concentrations ranged between 4.8- 12.1 (normal range 3.3-5.6). No patient was hypoglycemic during the ammonia load .

No differences in plasma and CSF ammonia and amino acid concentrations could be found whether the ammonia test lasted 3 or 6 hours.

The patients did not react very much to the ammonia load. Some of them felt a little nausea immediately after intake . Only two patients (nr 3 and 6) reported dizziness of short duration. No other mental disturbances could be observed during the test.

* 8 normal healthy volunteers.

DISCUSSION

Several etiological factors have been suggested to contribute to HE. The oldest known and most widely discussed is ammonia. The correlation between the grade of HE and the plasma concentrations of ammonia is, however, not very good suggesting that ammonia may not only have a direct effect on the central nervous system, but it may also act synergistically with or via other factors (16). Disturbances in the metabolism of the neurotransmitters, secondary to an accumulation of their large neutral amino acid (LNAA) precursors, have been suggested to contribute to HE (17). In a "unified hypothesis" James et al (1) linked the neurotransmitter changes to hyperammonemia. They proposed that high concentrations of glutamine, secondary to ammonia detoxification in brain, increased the transport of LNAA into brain and thereby influenced the neurotransmitter metabolism and contributed to HE. Increased blood-brain transport of LNAA has been demonstrated in rats after PCA (18) and in humans with liver cirrhosis (19). Infusion of ammonium salts in rats resulted in increased blood-brain transport and increased brain concentrations of these amino acids (2,3). It has not been demonstrated, however, that high ammonia concentrations can increase the brain concentrations of the LNAA in humans.

The etiology of reduced plasma concentrations of BCAA often seen in liver disease is unknown. Hyperinsulinemia has been suggested to reduce the plasma concentrations of BCAA (6) but other investigators have not been able to confirm this (7), suggesting that other factors are responsible for the reduced plasma levels of BCAA. In vitro experiments

have demonstrated a connection between the peripheral ammonia and BCAA metabolism (8,9). BCAA is transaminated with alpha-ketoglutarate to form glutamic acid. Glutamic acid can be further aminated in the presence of ammonia to form glutamine. In accordance, intraperitoneal injection of ammonium acetate into rats with portacaval anastomosis reduced the plasma concentrations of BCAA (1). In one study on humans with liver cirrhosis it has been suggested that ammonia had the same effect on BCAA (10).

In the present study, the plasma and CSF ammonia concentrations increased concomitantly after administration of ammonium chloride (Fig 1). However, no parallel increase in the glutamine and ammonia concentrations was found in plasma or in CSF (Fig. 1,2,4). To study if the duration of the ammonia load was too short we prolonged the ammonia test in three patients from three to six hours. No difference was found between the three hours and the six hours ammonia tests. After the ammonia load there was a marked increase in the CSF concentrations of glutamine and LNAA in only one patient (number 5) (Fig. 4 and 5). This patient was the only one who exhibited pronounced encephalopathy (Table 1). It is remarkable, however, that his basal glutamine levels seemed to be lower than those of the other patients. One explanation of the lack of effect of ammonia load on the other patients could be that their liver function was less impaired although there were few disturbances in the liver function tests of the patients overall. Another reason for the differences in response to the ammonia load may be that patient number five still consumed alcohol regularly. The repeated toxic effect of alcohol may increase his sensitivity to ammonia and contribute to encephalopathy.

Glutamic acid can donate its amino group to pyruvate, forming alanine, or it can be further aminated to form glutamine in the presence of ammonia (8,9). The plasma alanine concentrations, however, increased in the patient who showed the highest increase in the plasma glutamine concentrations after the ammonia load (Fig. 2). The plasma concentrations of glutamic acid was slightly reduced in six of the tests (Fig 3).

The absence of any major effect of an ammonia load on amino acids in CSF in the present experiment is in contrast to many studies on experimental animals (1,2,3). Different species may react in a different manner, and similar experimental works have not been performed on animals with liver cirrhosis. Metabolic events in the brain tissue may not directly be reflected in the CSF. Thus, comparisons between CSF and brain tissue concentrations must be interpreted with caution. However, a good correlation was demonstrated between the

CSF glutamine concentrations and the CSF concentrations of phenylalanine, tyrosine, and methionine (Table 2). This observation may support that also in humans the glutamine concentrations could influence the concentrations of the other LNAA. The poor correlation between the CSF glutamine concentrations and the CSF concentrations of valine, isoleucine and leucine could be explained by the low affinity of valine and isoleucine to the LNAA transport system (20) and to the relatively rapid metabolism of leucine in brain (21). The same relationship between the brain glutamine concentrations and the brain concentrations of these amino acids has been demonstrated in rats with PCA (22).

After administration of ammonia we found an increase in the plasma concentrations of phenylalanine, tyrosine and the BCAA only in one patient. The etiology of his liver cirrhosis was sarcoidosis, while the other patients had alcohol cirrhosis. This patient was also the only one who had a great increase in the plasma glucagon concentrations after administration of ammonium chloride. The etiology of the liver cirrhosis may be of importance for the reaction to ammonia.

Marchesini et al (10) have performed ammonia tolerance tests in 17 patients with advanced liver cirrhosis. They found a rise in the plasma concentrations of phenylalanine, tyrosine, glucagon and insulin and a reduction of the BCAA concentrations. One explanation of the different results in the two investigations may be a more advanced liver disease in the patients of the Italian study. Eight patients in the Italian study had ascites while no patient had ascites in our study. Moreover, encephalopathy seemed to be more pronounced in the study by Marchesini et al. Another explanation could be a different design of the ammonia tolerance tests. Marchesini et al gave one single dose of 3 g of ammonium chloride and drew blood samples after 45 minutes, whereas we gave repeated doses of 2 g of ammonium chloride during a longer period. No information could be found in the study by Marchesini et al whether the etiology of the cirrhosis influenced the results.

High concentrations of indoleamines and tryptophan have been demonstrated in rats with PCA (23) and after hepatectomy (24). High CSF concentrations of tryptophan and 5-HIAA have been demonstrated in humans with HE (25). The tryptophan concentrations in CSF were not determined in the present work but a good correlation was observed between the concentrations of 5-HT and 5-HTP and the glutamine concentrations in CSF (Table 2). This may suggest that glutamine stimulates the uptake of tryptophan into brain and, consequently, influences the indoleamine metabolism.

The results of the present study highlight the difficulties in applying results from

experimental animals to humans. We could not demonstrate an increase of LNAA in CSF after ammonia intake in patients with liver cirrhosis and a history of HE. However, ammonia could have such effects in patients with a more severe degree of liver insufficiency. The present study demonstrates that the etiology of liver cirrhosis and the degree of liver function must be clearly defined before we can discuss differences or similarities between studies. The possible influence of ammonia on glutamine and amino acid concentrations in CSF may be slow, which has to be considered when evaluating new therapeutic modalities affecting these mechanisms. Further studies with ammonia tolerance tests in patients with severe liver disease and HE are needed to clarify the interaction of ammonia, LNAA, glucagon and insulin.

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Table 1. Clinical and Laboratory Data on the Patients.

Pat nr	Age yrs	First bleeding	Treatment	Etiology of liver cirrhosis	Albumin * g/l	Prothromb. ^a ** index	ASAT ^b *** μ kat/l	Psychologic test
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3 hour ammonia test

1	65	jan 81	sclerotherapy	alcohol	36	50	0.6	normal
2	52	nov 78	shunt ^c jul 78	" "	34	26	0.6	normal
3	56		shunt ^c oct 84	" "	35	45	1.0	slight enc. ^d
4	69	oct 79	shunt ^c feb 80	sarcoidosis	28	50	1.1	slight enc. ^d
5	59	nov 80	shunt ^c dec 80	alcohol	38	45	0.7	pronounced enc. ^d

6 hour ammonia test

6	65	jan 81	sclerotherapy	alcohol	36	50	0.6	normal
7	41	apr 83	shunt jan 84	" "	38	50	1.0	slight enc. ^d
8	44	apr 84	shunt sept 84	" "	34	>50	0.6	slight enc. ^d

*normal range 36-48g/l; **normal range 70-130 %; ***normal range <0.67 μ kat/l.

Patient number 1 and 6 is the same.

^a Prothromb = prothrombin ; ^b ASAT = aspartate-aminotransferase; ^c shunt = mesocaval interposition shunt; ^d enc. = encephalopathy

Table 2. Linear Correlation between the Gutamine Concentrations and the Concentrations of other LNAA and 5-HT and 5-HTP.

	r
Valine	0.11
Isoleucine	0.27
Leucine	0.40
Phenylalanine	*0.90
Histidine	*0.90
Methionine	*0.92
5-HT	*0.86
5-HTP	*0.88

n = 16;

r = correlation coefficient; * $p < 0.001$

LEGENDS

Figure 1. Ammonia concentrations in plasma and CSF before and after ammonia tolerance tests. Shadowed area indicates normal range. Patients nos 1-5 underwent three hours and patients nos 6-8 six hours ammonia tolerance test, respectively. Psychological tests revealed normal mental status in patients nos 1,2 and 6, slight encephalopathy in nos 2,3,4,7 and 8, and pronounced encephalopathy in no 5. All patients except nos 1 and 6 were operated upon with a mesocaval shunt.

Figure 2. Plasma glutamine and alanine concentrations before and after ammonia tolerance tests. Shadowed area indicates normal range. For description of patients and treatment see legend figure 1.

Figure 3. Plasma glutamic acid concentrations before and after ammonia tolerance tests. Shadowed area indicates normal range. For description of patients and treatment see legend figure 1.

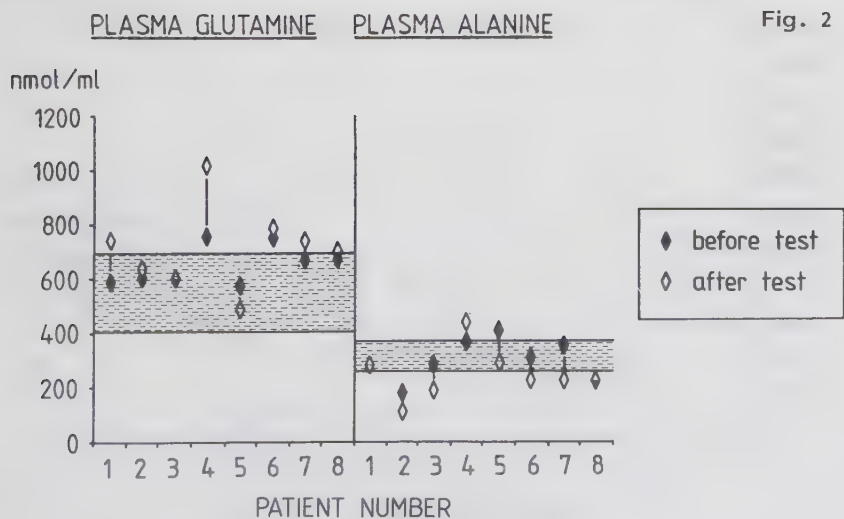
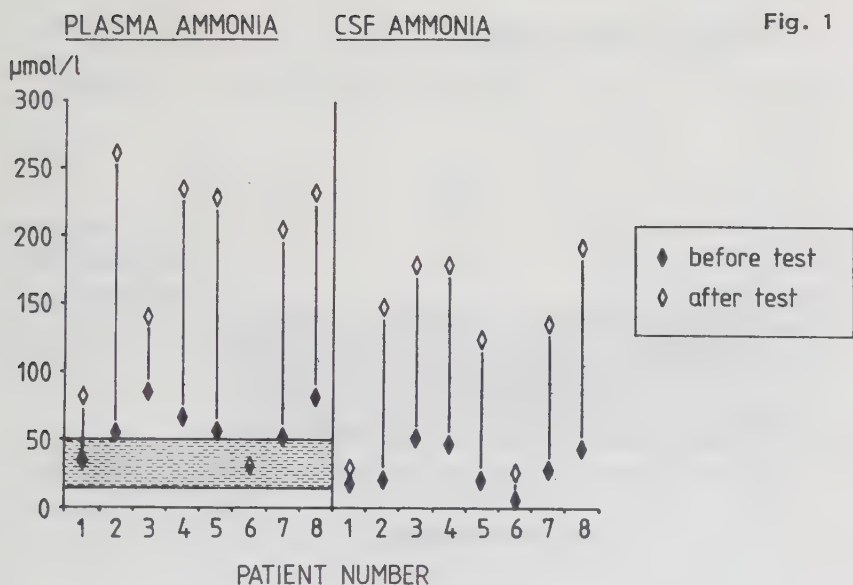
Figure 4. CSF glutamine concentrations before and after ammonia tolerance tests. For description of patients and treatment see legend figure 1.

Figure 5. CSF concentrations of valine, isoleucine, leucine (5A), phenylalanine, tyrosine and methionine (5B) before and after ammonia tolerance tests. For description of patients and treatment see legend figure 1.

Figure 6. Plasma concentrations of valine, isoleucine, leucine (6A), phenylalanine, tyrosine and methionine (6B) before and after ammonia tolerance tests. Shadowed area indicates normal range. For description of patients and treatment see legend figure 1.

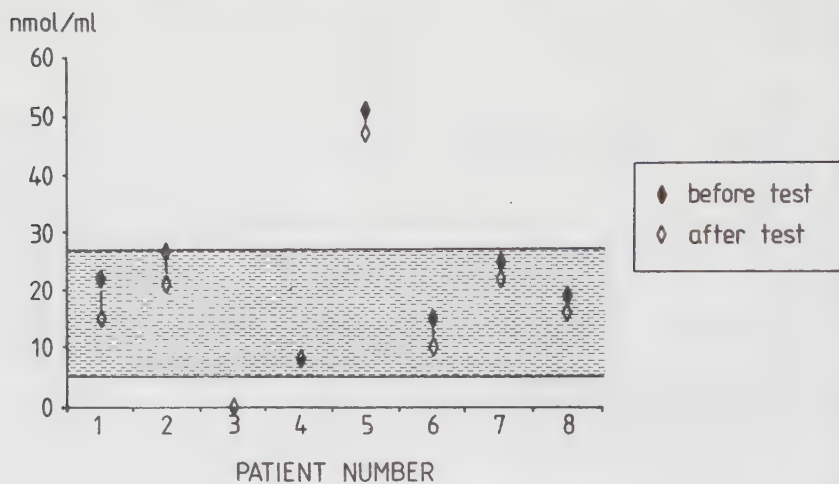
Figure 7. CSF concentrations of 5-hydroxytryptamine (5-HT), 5-hydroxytryptophan (5-HTP)(7A) and 5-hydroxyindoleacetic acid (5-HIAA)(7B) before and after ammonia tolerance tests. For description of patients and treatment see legend figure 1.

Figure 8. Plasma concentrations of glucagon and insulin before and after ammonia tolerance tests. Shadowed area indicates normal range. For description of patients and treatment see legend figure 1.



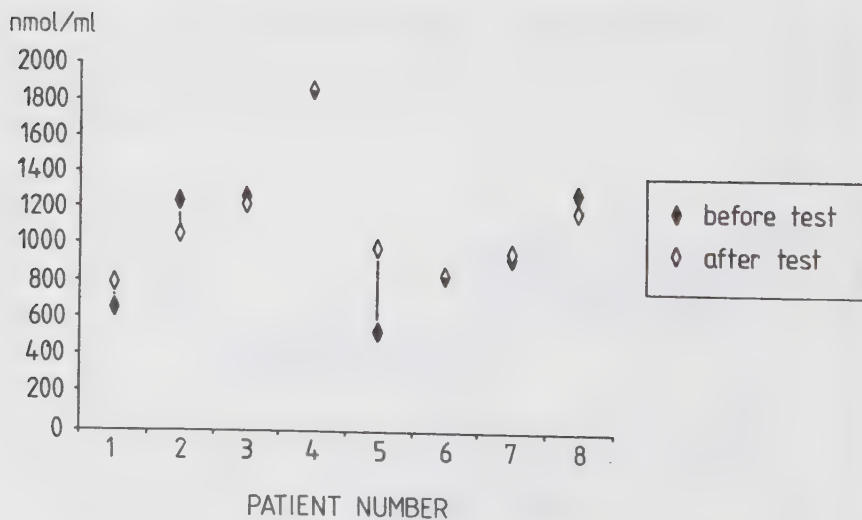
PLASMA GLUTAMIC ACID

Fig. 3



CSF GLUTAMINE

Fig. 4



CSF

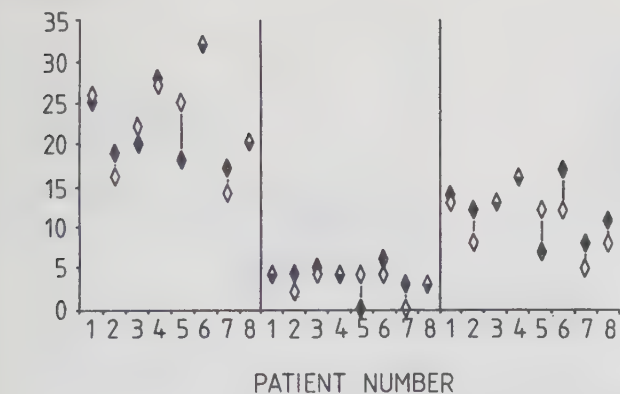
Fig. 5A

VALINE

ISOLEUCINE

LEUCINE

nmol/ml



◆ before test
◇ after test

CSF

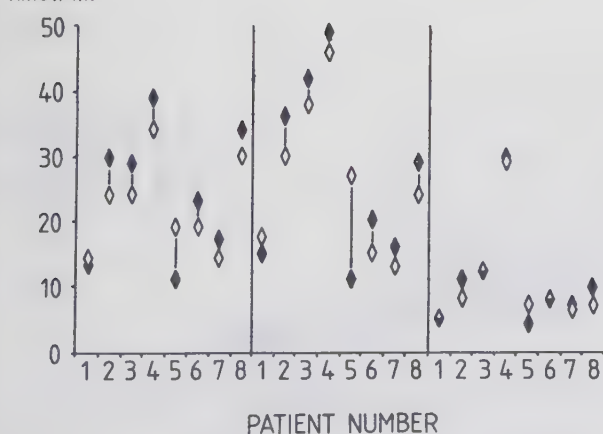
Fig. 5B

PHENYL-
ALANINE

TYROSINE

METHIONINE

nmol/ml



◆ before test
◇ after test

PLASMA

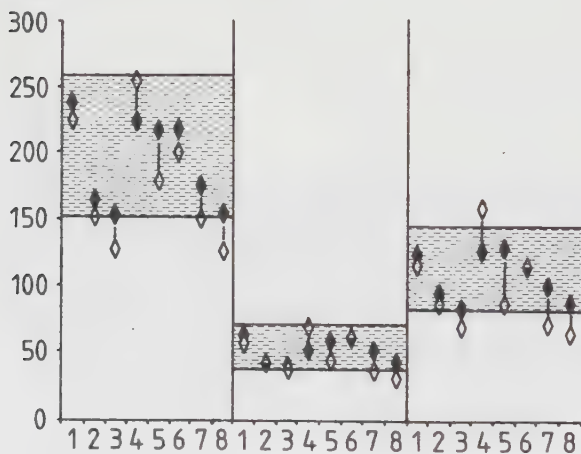
VALINE

ISOLEUCINE

LEUCINE

Fig. 6A

nmol/ml



◆ before test
◇ after test

PATIENT NUMBER

PLASMA

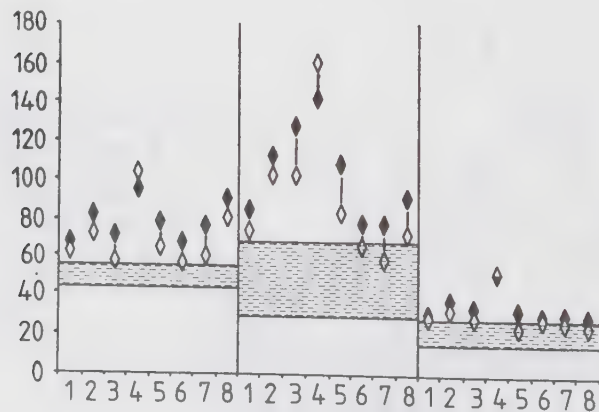
PHENYL-
ALANINE

TYROSINE

METHIONINE

Fig. 6B

nmol/ml



◆ before test
◇ after test

PATIENT NUMBER

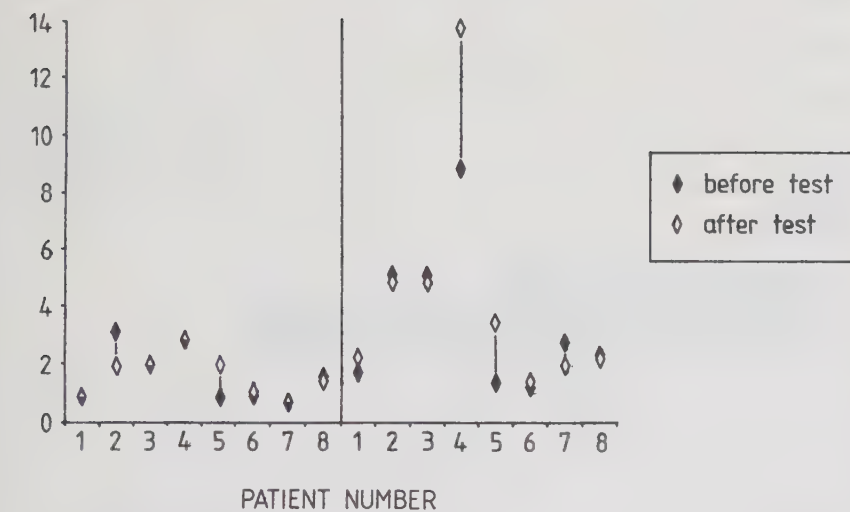
CSF

Fig. 7A

5-HT

5-HTP

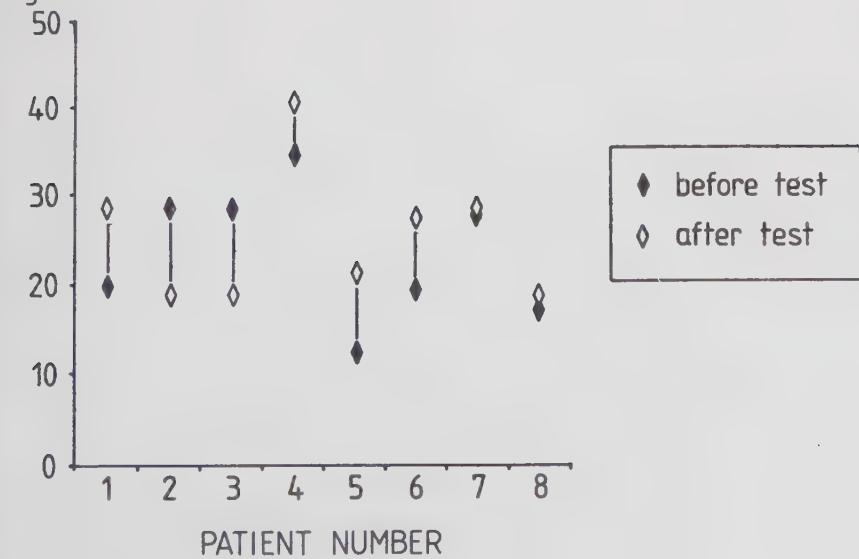
ng/ml



CSF 5-HIAA

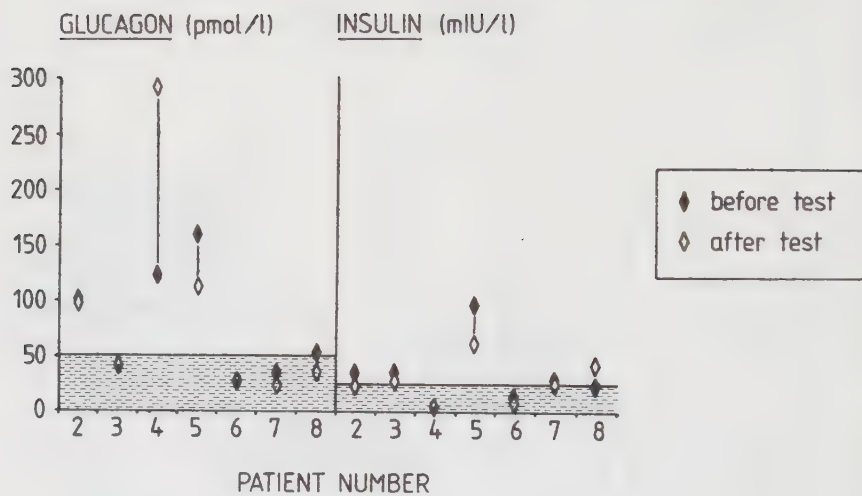
Fig. 7B

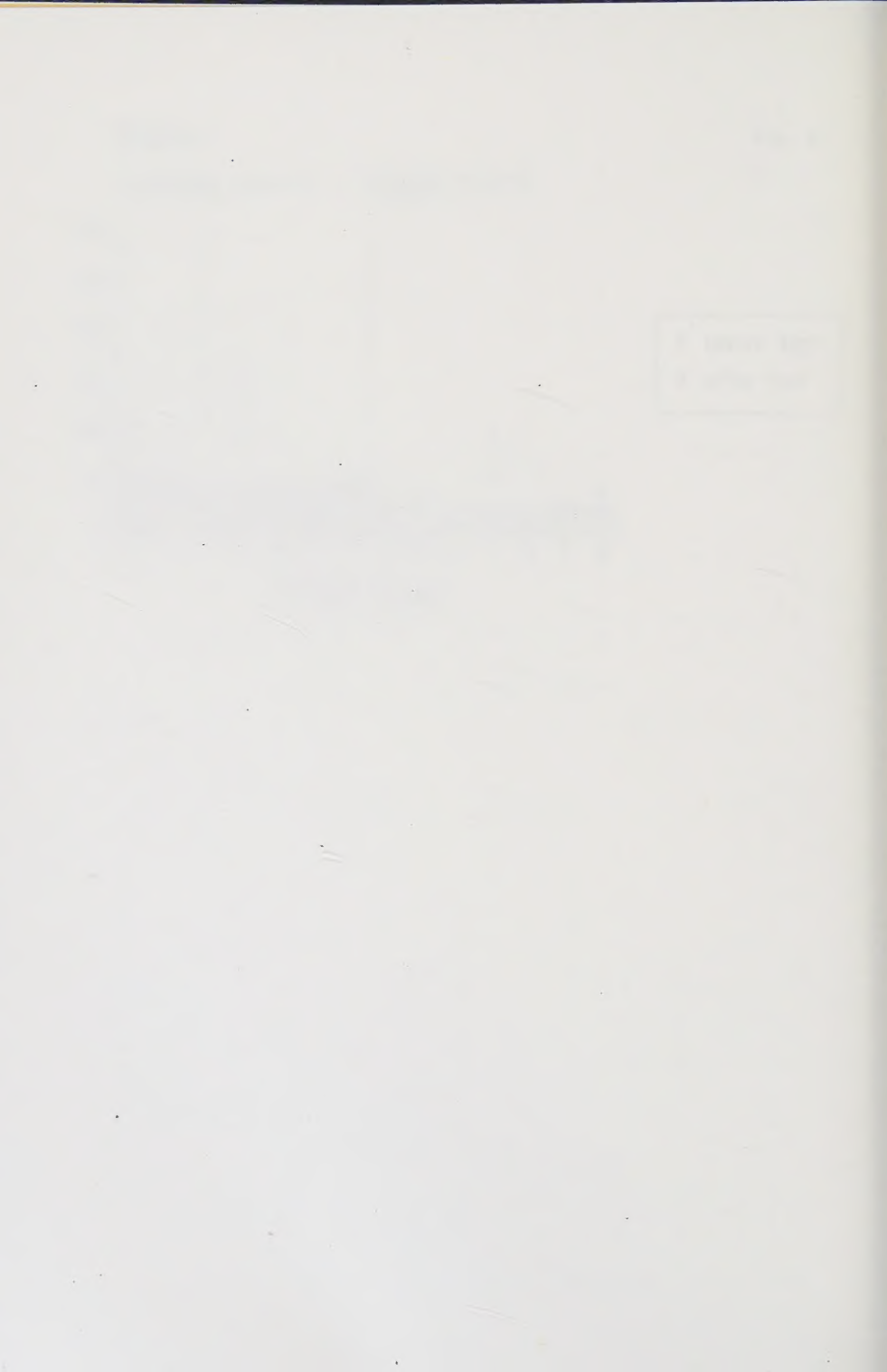
ng/ml



PLASMA

Fig. 8





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